Amended claim 14 substitutes the word "methionine" for the term "initiation codon," for consistency with the above clarification. The word "methionine" in this context is understood by the skilled artisan to mean a methionine encoded by an initiation codon (i.e. an ATG codon) as described at paragraph 37 of the substitute specification (i.e page 11, lines 31-33, of the original specification) and recited in original claim 14.

Claim 15 is amended to a dependent claim. Support for amended Claim 15 is to be found in original Claims 14 and 15.

Claim 16 is amended to require at least three, rather than two, CTL epitopes. Support for amended claim 16 is to be found *inter alia* in the description at paragraph 10 of the substitute specification filed February 20, 2002 (i.e. at page 2, lines 33-34, of the specification as originally filed).

Claim 17 is amended to require four, rather than three, CTL epitopes. Support for amended claim 17 is to be found *inter alia* in the description at paragraph 74 of the substitute specification filed February 20, 2002 (i.e. at page 22, lines 20-22, of the specification as originally filed).

Claim 33 is amended to include the additional requirement that each CTL epitope of the polynucleotide of the claimed vaccine is substantially free of sequences found naturally to flank that CTL epitope. Support for this feature is to be found *inter alia* in the final clause of pending claim 14 and in the description at paragraph 5 of the substitute specification filed on February 20, 2002 (i.e. corresponding to the description at page 2, lines 1-9 of the original specification).

Amended claim 33 also clarifies that it is the CTL epitopes encoded by the nucleic acid vaccine, rather than the nucleic acid *per se*, that are either contiguous or spaced apart by an

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intervening sequence that does not comprise a methionine. Support for contiguous CTL epitopes is to be found *inter alia* in Figure 1 and Figure 5 of the application as originally filed. Support for the use of intervening sequences is to be found *inter alia* in Figure 5 of the application as originally filed, wherein a spacer sequence TS is positioned between the epitopes YPHFMPTNL and SGPSNTPPEI.

Amended claim 33 substitutes the word "methionine" for the term "initiation codon," for consistency with the above clarification. The word "methionine" in this context is understood by the skilled artisan to mean a methionine encoded by an initiation codon (i.e. an ATG codon) as described at paragraph 37 of the substitute specification (i.e page 11, lines 31-33, of the original specification) and recited in original claim 14.

Claim 34 is amended to a dependent claim. Support for amended Claim 34 is to be found in original Claims 33 and 34.

Accordingly, no new matter has been added by the present amendments. Applicants respectfully request the Examiner to enter the amendment.

B. Status of the Claims

Claims 14-34 were pending at the time of the Action. Claims 14-17, 33 and 34 have been amended. A copy of the amended claims with editing indicia is attached as exhibit B. A clean copy of the presently pending claims is attached as Appendix C.

Applicants also note that at paragraph 2 of the Office Action mailed on May 21, 2002, the Examiner points out that the amendment filed February 20, 2002, indicated the introduction of new claim 35, however, no such claim was included in the amendment. In response to this objection, Applicants advise that there is no intention at present to introduce claim 35 to the application. Applicants respectfully request the Examiner to disregard references to Claim 35 in

the papers filed February 20, 2002. Applicants apologize for any inconvenience caused to the Examiner by the erroneous reference to Claim 35 in the papers filed. Therefore, no claim 35 is currently pending.

C. Objection to the Drawings

At paragraph 4 of the Office Action mailed on May 21, 2002, the Examiner indicates that the drawings filed May 22, 2000 stand not approved.

In consideration of the matters raised in PTO 948, Notice of Draftperson's Patent Drawing Review, Applicants submit herewith substitute drawings sheets 1/29 through 29/29 which the applicants submit meet formal requirements. Please substitute the drawings attached at Appendix D for the informal drawings presently on file.

Reconsideration and withdrawal of the objection is respectfully requested in view of the substitute drawings.

D. Objection to the Specification

At paragraph 5 of the Office Action mailed May 21, 2002, the Examiner has objected to the disclosure on the basis that there is no SEQ ID NO: reference at page 19, line 6, lines 9-10 and lines 14-15.

In response to this objection, Applicants have amended the specification to recite appropriate SEQ ID NO: listings on page 19, line 6, lines 9-10, and lines 14-15 (i.e. paragraphs 73 and 74).

Reconsideration and withdrawal of the objection is respectfully requested in view of the amendment.

E. Claims 14-34 are enabled under 35 U.S.C. §112, First Paragraph

At paragraphs 6 and 7 of the Office Action mailed on May 21, 2002, the Action has maintained the rejection of Claims 14-34 under 35 U.S.C. 112, first paragraph, on the basis that the specification, while enabling for a recombinant vaccine CTL polyepitope-based composition comprising a polynucleotide encoding CTL epitopes as depicted in Figure 5 derived from pathogens MCMV, influenza, EBV, Adenovirus, and EG7 tumor for use as vaccines, does not reasonably provide enablement for vaccine compositions and their use in vaccination against any HIV.

In maintaining this rejection, the Action states that the only disclosed use of the claimed polynucleotides in claims 14 to 32 is for a vaccine which by definition is for the prevention of a particular disease. Accordingly, the Action considers that the rejection is equally applicable to claims 14-32 directed to polynucleotides and to claims 33 and 34 directed to nucleic acid vaccines.

Applicants respectfully traverse this rejection and submit herewith an executed Declaration by the inventor SUHRBIER and Exhibits AS1 through AS16 for consideration (Appendix E). In particular, Dr Suhrbier attests to the fact that, while useful for producing a vaccine composition, the claimed polynucleotide has additional uses, such as, for example, the generation and monitoring of CTL responses (*see* paragraphs 13-17 of the Suhrbier Declaration). We particularly direct the Examiner's attention to the fact that, as noted at paragraph 15 of the Suhrbier Declaration, several U.S. patents have been granted for isolated HIV-1 CTL epitope peptides on applications filed before the priority date of the instant application (i.e. U.S. Patent No 5,700,635 to McMichael *et al.;* U.S. Patent No 5,932,218 to Berzofsky *et al.*; and U.S. Patent No 6,294,322 to Berzofsky *et al.*).

"U.S. patents are considered pertinent evidence of what is likely to be known by persons of ordinary skill in the art." *In re Howarth,* 654 F.2d 103, 107 (CCPA 1981). Accordingly, it was known by those skilled in the relevant art before the priority date of the instant application that CTL epitope peptides have utilities extending beyond vaccine preparations, including assays for CTLs and enhancing CTL responses.

At paragraphs 18 through 22 of the Suhrbier Declaration, Dr Suhrbier also directs attention to the fact that the Action erred in stating that the only *in vivo* working example provided in the specification is of the polyepitope provided in example 2. In fact, Example 1 of the specification as originally filed also provides support for a polyepitope construct using human epitopes.

With respect, it is incorrect to assume that the only disclosed utility for the subject polynucleotide constructs is as a vaccine because, the specification clearly discloses other uses, such as CTL assays and the enhancement of CTL responses. Applicant also directs the Examiner's attention to the description at page 9, line 3 to page 10, line 6 of the substitute specification filed February 20, 2002, which clearly teaches the use of human polyepitope-encoding construct to produce CTL clones capable of killing human HLA-matched target cells, and to stimulate *in vitro* secondary CTL responses from peripheral blood mononuclear cells (PBMC), and to recall epitope-specific CTL responses. Moreover, the description at page 13 (i.e. paragraph 50) discusses use of the mouse polyepitope construct to induce primary CTL responses in mice. Proceeding on this basis, Applicants again request the Examiner to reconsider and withdraw this rejection of Claims 14-32.

At paragraphs 23 through 29 of the Suhrbier Declaration, Dr Suhrbier notes that even at the priority date of the instant application, several CTL epitopes had been isolated from a range

of different antigens, and methods were well-established for determining the structure and function of any CTL epitope. Accordingly, it was routine at that date to provide functional CTL epitopes for inclusion in a polyepitope produced in accordance with the present invention. With respect, given the numerous examples of individual CTL epitopes in the literature at that time, a skilled molecular biologist would readily produce any number of polyepitope-encoding constructs as presently claimed without undue experimentation. The invention resides in the presentation of a chimeric gene encoding a plurality of CTL epitopes as a single fusion polypeptide and not in the structure of any particular CTL epitope.

Applicants respectfully submit that given the working examples of such polyepitope-encoding constructs provided in the specification and the routine nature of identifying CTL epitopes at the priority date, the specification clearly enables a skilled person to produce any polyepitope-encoding construct independent of the structure of any CTL epitope. In support of this conclusion, Applicants further direct the Examiner's attention to paragraph 33 of the Suhrbier Declaration exemplifying several polyepitope-encoding constructs produced according to the teaching provided in the specification.

With regard to the rejection of Claims 33 and 34, Applicants also direct the Examiner's attention to the paragraphs 34 to 37 of the Suhrbier Declaration. In particular, Dr Suhrbier points out at paragraph 35 that example 2 of the instant specification demonstrated CTL responses to a wide range of different pathogens, indicating that the pathogen *per se* was not a limiting factor in producing a protective immune response.

Moreover, data on protection are not the only means of assessing the efficacy of a vaccine. For human HLA A2-restricted CTL epitopes, mouse models (i.e. HHD or HLA A2/K^b transgenic mice) can be used to determine protection. For other human CTL epitopes, it is well

known to determine CD8+ T-cell induction by the polyepitope, or cross-recognition with CD8+ T-cells from infected patients, as an indicator of vaccine efficacy, as stated in the Suhrbier Declaration. Thus, data on protection obtained from clinical trials are not essential as suggested by the Action. Applicants respectfully point out that example 1 of the specification teaches cross-recognition of each epitope of the expressed polyepitope with CD8+ T-cells from EBV-infected patients, suggesting efficacy of the subject construct as a nucleic acid vaccine.

Applicants respectfully submit that no undue experimentation would be required to practice the invention as claimed in claims 14-32 in any event, but most certainly in light of the proper content and meaning of the claims as perceived by those of skill in the relevant art. See the declaration of Dr. Suhrbier paragraphs 15-41 and the declaration of Mr. Cox attached as Appendix F at paragraph 41. Specifically, Applicants respectfully submit that the citation to Ramsay et al. (1992-1994) was not current with the state of immunological research into HIV protect as of 1997 and is certainly not applicable to the successes achieved as provided by the declaration of Dr. Suhrbier. Specifically, see the declaration of Dr. Suhrbier at paragraphs 33-36 which sets out numerous references to the successes of polytope constructs within the scope of the present invention.

See, especially, paragraph 36 of the declaration of Dr. Suhrbier, which relates that "Gardner et al. teach a polyepitope construct encoding multiple tandem HIV HLA A2-restricted CTL epitopes (Table 1), its delivery by modified vaccinia virus Ankara (MVA), and the testing of same in HLA A2 transgenic mice prior to human clinical trials (p. 296 "Polytope vaccines against HIV/AIDS"). Gardner et al. state at page 296, lines 1-5, that **the "polytope approach has now been shown to work for a variety of epitopes, diseases and vectors including HIV** and Modified Vaccinia Ankara (MVA) (Hanke et al., 1998), malaria and Ty particles (Gilbert et

al., 1997), different viruses and vaccinia (An and Whitton, 1997), influenza and lipopeptides (Sauzet *et al.*, 1995) and tumors and recombinant adenovirus (Toes *et al.*, 1996)." Emphasis added.

These successes are in complete accord with the expectations of the artisan in light of the knowledge of the immunological arts: "Given the plethora of known CTL epitopes from HIV at the filing date of The Present Application, and from the teaching provided that individual CTL epitopes are correctly processed and presented from the claimed polyepitope formulation, I conclude that it would be a routine matter to substitute the exemplified CTL epitopes provided by the inventors with other CTL epitopes, specifically known HIV-1 CTL epitopes. Accordingly, the specification as originally filed reasonably conveys that the inventors were in possession of polynucleotides encoding polyepitope polypeptides comprising CTL epitopes from a wide range of pathogens." Declaration of Mr. John Cooper Cox at paragraph 41.

In contrast to the statements of those of skill in the art, and the now documented success of the polytope constructs in producing the desired immune responses when used for that purpose, the Action points to no scientific reference or principle the sustain the position that when HIV epitopes are chosen to be incorporated into a polytope construct of the present invention such a construct could not successfully be so made and used.

Applicants submit that the instant rejection for lack of enablement is improper. Reconsideration and withdrawal of the rejection is respectfully requested.

F.   **Claims 14-34 satisfy the written description requirement of 35 U.S.C. § 112, first paragraph**

The Action rejects claims 14-34 under 35 U.S.C. § 112, first paragraph. The Action alleges that there is not adequate written description provided in the specification. In particular, the Action correctly notes that there are numerous CTL epitopes available for inclusion in a

polytope construct, both where the CTL epitopes are restricted by different or identical HLAs. However, the Action goes on to incorrectly conclude that the Applicants have provided an insufficient number of exemplary species to allow adequate description of the claimed genus of CTL epitopes. Applicants respectfully traverse.

The Action argues that the diversity of available CTL epitopes precludes a successful written description but for an exhaustive listing of all possible epitopes, the nucleotide sequences that encode them, and all combinations thereof. Such an exhaustive listing of all possible CTL epitope sequences is not required to meet the legal standard of written description. That the range of possible CTL epitopes is wide does not preclude adequate description. See *In re Angstadt*, 537 F.2d 498 at 502- 03, 190 USPQ 214, 218 (CCPA 1976) (Applicants "are <u>not</u> required to disclose <u>every</u> species encompassed by their claims even in an unpredictable art.")

Patent law, as set forth by the Federal Circuit and the MPEP, requires only that "an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics… i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." See Written Description Guidelines 66 Fed. Reg. at 1106, given judicial notice in *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 296 F.3d 1316, 1324 (Fed. Cir. 2002).

CTL epitopes are recognized by one of skill in the art of immunology and its related fields, who would understand that the specification of particular epitopes does not limit the scope of the disclosed invention. See the declaration of John Cooper Cox at paragraphs 10-17. In particular, Applicants respectfully note that the identification of CTL epitope from any source was a matter of routine at the time of invention and that the artisan had available a number of

methods by which to so identify and obtain epitopes as contemplated by the present invention. See, for example, paragraph 13 of the declaration of John Cooper Cox, which begins "Before the filing date of The Present Application, I was also aware of several well-established methods for determining CTL epitopes from any source." Mr. Cox then lists a number of methods, well known to those of skill in the art, which allow the routine identification of CTL epitopes from any source. Paragraph 13, Cox declaration.

Applicants respectfully submit that the identification of CTL epitopes as of the time of filing was a procedure in the immunological arts not unlike the generation of antibodies capable of binding a specific immunogen. In discussing the Written Description Guidelines, the PTO's controlling Court has noted that an "isolated antibody capable of binding to antigen X" is a claim that would satisfy the Written Description requirements of § 112 "notwithstanding the functional definition of the antibody, in light of the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature." *Enzo Biochem* at 1324-25. Applicants respectfully note that the functional and structural characteristics of CTL epitopes are well known and, that as of the time of the present filing, the field was mature with respect to the identification of epitopes for use within the context of the polytope constructs claimed. Declaration of John Cooper Cox, paragraphs 9-15.

Applicants respectfully submit that they have described more than a sufficient number of representatives of the genus of CTL epitopes within the context of the methods of the invention so as to demonstrate that they have fully set forth and possess the invention.

In *Enzo Biochem* at 1327 the Court cited *In re Smythe,* 480 F.2d 1376, 1383, 178 USPQ 279, 284-85 (CCPA 1973), which states that "We cannot agree with the broad proposition ...

that in every case where the description of the invention in the specification is narrower than that in the claim there has been a failure to fulfill the description requirement in § 112. Each case must be decided on its own facts. The question which must be answered is whether the application originally filed in the Patent Office clearly conveyed in any way to those skilled in the art, to whom it is addressed, the information that appellants invented the [claimed invention]" citing *In re Ruschig*, 54 CCPA 1551, 379 F.2d 990, 154 USPQ 118 (1967).

Here, Applicants respectfully submit that under the applicable legal standards, and in view of the factual knowledge of the artisan as exemplified in the declarations of John Cooper Cox and Dr. Suhrbier, Applicants have clearly conveyed to those of skill in the relevant art that Applicants have invented the claimed invention.

The enclosed COX Declaration and Exhibits JCC1 through JCC17 (i.e. Appendix F affixed hereto) provide objective evidence that the specification as filed contains sufficient representative examples to demonstrate to a skilled person that the inventors actually invented what they now claim.

For example, the Cox Declaration provides the following responses of one of skill in the art to the allegations of the Action:

(i) THAT the constructs provided by the specification are generic for constructs comprising two or more CTL epitopes, and, given the large number of CTL epitopes available at the filing date, a skilled researcher in the field of vaccine delivery, bacteriology and virology, desirous of constructing a composition for use as a vaccine, would understand that the selection of appropriate epitopes for any particular vaccine application, and the incorporation of such epitopes into a polytope construct as provided

by the inventors, would be routine in view of the skill in the arts and the guidance provided by the specification;

(ii)   THAT the exact CTL epitopes incorporated into the constructs of the invention are not limiting because the invention concerns polytope constructs that contain two or more appropriate CTL epitopes lacking naturally occurring flanking sequences or methionine residues, wherein the encoded polytope protein is successfully processed to the individual component CTL epitopes which are expressed on the APC surface, induce CTL responses, and are recognized by pre-existing or induced CTL to cause killing of infected cells;

(iii)   THAT the skilled artisan would readily appreciate from the teachings of the specification that such polytope constructs could comprise any number of contiguous or spaced CTL epitopes that do not include naturally occurring flanking sequences or methionine residues;

(iv)   THAT the skilled artisan would find the manner of making the constructs of the present invention routine in view of the guidance provided in the specification, because the materials and the procedures for constructing the polyepitope constructs were readily available to the artisan at the time the application was filed;

(v)   THAT *both* examples 1 and 2 of the application as originally filed show that the novel human and mouse constructs do, in fact, work, *i.e.*, that CTL epitopes joined as directed by the specification are effectively processed, presented, and effective in inducing CTL responses to each epitope encoded by the polypeptide construct; and

(vi)   THAT the skilled artisan would also appreciate that the examples provided are not limiting, and that the type of polytope construct as claimed by the Inventors would work for any combination of CTL epitopes.

With respect, based on the wealth of publicly available information at the time the application was filed and the representative number of working examples provided in the specification as filed, Applicants respectfully maintain that, in contrast to the Action's position, the specification as originally filed does adequately describe the present invention under the requirements of the first paragraph of 35 U.S.C. § 112.

Review and withdrawal of the rejection is respectfully requested in view of the foregoing remarks.

G.   No new matter is introduced by claims 14-34

At paragraph 9 of the Office Action mailed on May 21, 2002, the Action has maintained the rejection of Claims 14-34 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention.

In particular, the Action states that the specification as originally filed requires the CTL epitopes to be "substantially free of peptide sequences naturally found to flank the CTL epitopes." In response to this rejection by the Action, Applicants have herein above amended claims 14 and 33 to recite the feature that "each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope." Applicants respectfully submit that the amendment to these claims overcomes this new matter rejection.

The Action also maintains that the specification and claims as originally filed do not provide clear support for "at least two," "nine" and "ten" CTL epitopes. In response to this rejection, Applicants have amended Claims 16 and 17 to require "at least three" and "four" CTL epitopes, respectively. Support for the amended claims is to be found *inter alia* in the description at page 2, lines 33-34, and at page 22, lines 20-22, of the specification as originally filed. As for claims 18 and 19, Applicants maintain that examples 1 and 2 of the application as originally filed provide clear and adequate support for this number of CTL epitopes, as attested in the accompanying Cox Declaration.

The Action also states that there is no support in the specification and claims as originally filed for the term "viral vector" in Claim 21, because the specification merely refers to vaccinia vectors, avipox virus vectors bacterial vectors, virus-like particles and rhabdovirus vectors. Applicants respectfully traverse this rejection by the Action on the basis that the disclosure at page 3, lines 13-26 of the specification as originally filed merely recites such vectors as examples of "various vectors, and cites the disclosures by Chatfield *et al.*, Taylor *et al.* and Hodgson as providing additional information regarding suitable vectors.

It is clear that the specification did not intend the list of vectors provided at page 3 of the specification to be exhaustive. See Appendix F, the Cox Declaration. Moreover, several other viral vectors, in addition to vaccinia vectors, avipox virus vectors, and rhabdovirus vectors, were well-known at the filing date to be useful for expressing recombinant proteins in mammalian cells, including their use as vaccine vectors. The enclosed Cox Declaration (Appendix F) supports the conclusion that the skilled artisan would have been aware at the filing date of a number of different viral vectors and that any known viral vector could be successfully used in the context of the present invention. Accordingly, a skilled artisan would, at that date, have

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considered the specification to read on to such other viral vectors, the use of which to express a given protein would have been routine. Applicants, therefore, respectfully submit that, based upon what was then known and the disclosure of three examples of viral vectors in the specification as filed, the specification satisfies the written description requirement with respect to viral vectors generally.

Reconsideration and withdrawal of the new matter rejections is respectfully requested in view of the foregoing amendment, remarks and the attached declarations.

H. Claims 20-24 are definite under the second paragraph of 35 U.S.C. § 112

At paragraphs 10 and 11 of the Office Action mailed on May 21, 2002, the Action has maintained the rejection of Claims 20-24 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the Applicants regard as the invention.

In particular, the Action states that the phrase "virus-like particle," as recited in claims 21 and 24, is ambiguous because it is not clear whether it means viral particles or DNA plasmid from virus, rather than vaccinia virus particles engineered to express the T cell epitopes.

Applicants respectfully note that with respect to rejected claims 20, 22 and 23, these claims do not recite "virus-like particle." Thus, there appears to be no extant grounds for rejection of claims 20, 22 and 23 in the instant case. Applicants, therefore, respectfully request that their rejection be withdrawn.

Moreover, with respect to claims 21 and 24, Applicants respectfully note that the phrase "virus-like particle" ("VLP") is sufficiently defined by the specification in light of the knowledge of the relevant artisan. See the declaration of John Cooper Cox at paragraphs 48-51, which clearly indicate that one of skill in the art finds the phrase clear and definite.

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Furthermore, it is well known that the term "VLP" refers to a non-replicative particle having one or more of the capsid proteins of a virus that are generally reconstituted *in vitro*, in the presence of heterologous RNA or DNA such that this nucleic acid is packaged therein. For example, virus-like particles have been synthesized for viruses of various families using a baculovirus-insect cell expression system. VLPs are known to serve as vehicles for the transfer of genetic material to a host cell (e.g. U.S. Patent No. 6,420,160).

With respect, it is apparent to the skilled artisan that VLP is not limited to a vaccinia virus particle engineered to express a polyepitope of the invention, as suggested by the Action. Clearly, any suitable VLP expression system could readily be employed in this manner at the filing date of the present application.

It is also apparent that a skilled artisan would not interpret the term "VLP" to mean an infectious and replicative viral particle (i.e. native virus) or isolated DNA from a virus, as suggested by the Action. This is because a VLP is always considered an entity that is distinct from the native virion.

With respect, in view of the numerous prior art disclosures of VLPs and their use in nucleic acid-based vaccines (*e.g.* U.S. Patent No. 6,420,160), the metes and bounds of claims 20-24 are clear.

Reconsideration and withdrawal of the rejection is respectfully requested in view of the foregoing remarks.

I.   **Claim 15 is novel under 35 U.S.C. § 102(b) over Whitton *et al.***

The Action rejects claims 14-16, 20-22, 25, 33 and 34 as anticipated by Whitton *et al.* (1993). Applicants respectfully traverse. Specifically, the Action states that Claim 15 recites a polynucleotide comprising a nucleic acid sequence encoding a plurality of CTL epitopes wherein

the sequence encoding the epitopes are contiguous, and that Whitton *et al.* teach a single polynucleotide comprising two CTL epitopes.

In response to this rejection, Applicants have herein above amended claim 15 to a dependent claim on Claim 14. Applicants have also amended claim 15 to recite that the epitopes, not the nucleic acid sequences, are contiguous. Applicants respectfully submit that the amendment renders this rejection moot. Reconsideration and withdrawal of the rejection is respectfully requested in view of the foregoing amendment to claim 15.

J. **Claims 14-16, 20-22, 25, 27, 33 and 34 are novel under 35 U.S.C. § 102(b) over Lawson *et al.***

At paragraph 14 of the Office Action mailed May 21, 2002, the Action rejects claims 14-16, 20-22, 25, 27, 33 and 34 as anticipated by Lawson *et al.* (1993). Applicants respectfully traverse. Specifically, the Action contends that Lawson *et al.* teaches a recombinant vaccinia expressing a full length HA polypeptide that inherently contains more than one CTL epitope.

Without conceding the correctness of the Action's position, Applicants have herein above amended claims 14 and 33 to recite that "each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope." Applicants respectfully submit that the amendments render this rejection moot. Reconsideration and withdrawal of the rejection is respectfully requested in view of the foregoing amendment to claims 14 and 33.

K. **Claims 14 and 17-21 and 23-32 are patentable under 35 U.S.C. § 103(a) over the cited references.**

At paragraphs 15 through 26 of the Office Action mailed May 21, 2002, the Action rejects Claims 14, 17-21, and 23-32 as being unpatentable over Lawson *et al.*, *J. Virol. 68*, 3505-2511, 1994, in view of Whitton *et al.*, *J. Virol 67*, 348-352, 1993, and in further view of

Berzofsky *et al,* or as being unpatentable over Lawson *et al. in view of* Latron *et al* or Burrows *et al* or Del Val *et al or* Panicali *et al* or Adams *et al* or Celis *et al* or Widman *et al.*

Applicants respectfully traverse these rejections in respect of the amended claims, on the basis that none of the citations mention or suggest polynucleotides encoding a plurality of CTL epitopes wherein each epitope is substantially free of sequences naturally found to flank the CTL epitopes. Reconsideration and withdrawal of the rejections is respectfully requested in view of the foregoing amendment to claims 14 and 33.

L.   Obviousness type double patenting

Applicants acknowledge the provisional rejection based upon obviousness type double patenting. Applicants will respond by submission of an appropriate terminal disclaimer upon the event that conflicting claims issue from the U.S. Application Ser. No. 09/576,107 and the present application.

M.   Conclusion

In view of the above, Applicants respectfully submit that the claims are in condition for allowance.   Applicants respectfully and earnestly request notification to that effect.   The Examiner is invited to contact the undersigned attorney at (512) 536-3043 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

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Date: November 21, 2002

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Suhrbier, *et al.*

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004USC1/TMB

Appendix A

MARKED UP COPY OF AMENDED SPECIFICATION PARAGRAPHS OF USSN 09/576,101

Underlining indicates insertion and [brackets] indicates deletion.

73.   To test this possibility mice (Balb/c) were infected with 10^4 pfu of Murine cytomegalovirus (MCMV) (K181 strain - Scalzo *et al.* 1995) and left for 5 weeks at which point strong CTL responses specific for the MCMV epitope, YPHFMPTNL (SEQ ID NO: 21), had developed (Scalzo *et al.* 1995 - Fig 2A). These mice were then given the murine polytope vaccinia and spleen cells assayed 10 days later for CTL specific for the three other epitopes presented by the polytope in this strain of mouse (RPQASGVYM (SEQ ID NO: 12), Lymphocytic choriomeningitis virus nuclear protein, H-2L^d; TYQRTRALV (SEQ ID NO: 20), influenza nuclear protein, H-2K^d and SYIPSAEKI (SEQ ID NO: 18), P. Berghei circumsporozoite protein, H-2K^d).

Results

74.   Responses to each of the three new epitopes was observed following polytope vaccination, illustrating that the YPHFMPTNL (SEQ ID NO: 21) specific CTL did not prevent priming of CTL specific for RPQASGVYM (SEQ ID NO: 12), TYQRTRALV (SEQ ID NO:

20), and SYIPSAEKI (SEQ ID NO: 18), when all four epitopes are presented together in the polytope. (Control animals receiving the human polytope vaccinia instead of the murine polytope vaccinia, showed only YPHFMPTNL (SEQ ID NO: 21) specific CTL).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Suhrbier, *et al.*	Group Art Unit: 1644
Serial No.: 09/576,101	Examiner: Huynh, P. N.
Filed: May, 22 2000	Atty. Dkt. No.: FBRC:004USC1/TMB
For: POLYEPITOPE VACCINES	

Appendix B

MARKED UP COPY OF AMENDED CLAIMS OF USSN 09/576,101

Underlining indicates insertion and [brackets] indicates deletion.

14.   (Amended) A polynucleotide comprising a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) [CTL] epitopes [,] wherein each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope and wherein at least two of [the sequences encoding said] the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence[s] that does not comprise a methionine [, wherein said intervening sequences do not (i) comprise an initiation codon or (ii) encode naturally occurring flanking sequences of the epitopes].

15.   (Amended) The [A] polynucleotide [comprising a nucleic acid sequence encoding a plurality of CTL epitopes,] of claim 14, wherein the [sequence encoding said] CTL epitopes are contiguous.

16.   (Amended) The polynucleotide of claim 14, wherein said polynucleotide encodes at least three [two] CTL epitopes.

17.   (Amended) The polynucleotide of claim 14, wherein said polynucleotide encodes four [three] CTL epitopes.

33.   (Amended) A nucleic acid vaccine comprising a polynucleotide comprising:
       (i) a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) [CTL] epitopes [,] wherein each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope and wherein at least two of [the sequences encoding said] the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence[s] that does not comprise a methionine [, wherein said intervening sequences do not (i) comprise an initiation codon or (ii) encode naturally occurring flanking sequences of the epitopes]; and
       (ii) an acceptable carrier.

34.   (Amended) The nucleic acid vaccine of claim 33 wherein the CTL epitopes are contiguous [A nucleic acid vaccine comprising a polynucleotide comprising a nucleic acid sequence encoding a plurality of CTL epitopes, wherein the sequences encoding said CTL epitopes are contiguous, and an acceptable carrier].

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Suhrbier, et al.	Group Art Unit: 1644
Serial No.: 09/576,101	Examiner: Huynh, P. N.
Filed: May, 22 2000	Atty. Dkt. No.: FBRC:004USC1/TMB
For: POLYEPITOPE VACCINES	

Appendix E

DECLARATION OF ANDREAS SUHRBIER AND EXHIBITS AS1-AS16

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
   Suhrbier, et al.

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

<u>DECLARATION OF ANDREAS SUHRBIER</u>

I, Andreas Suhrbier, hereby declare as follows:

1.   I am a German citizen residing at 185 Mailmans Track, Bunya, Queensland 4055, Australia.

2.   I am an inventor in respect of the above-captioned application ("The Present Application").

3.   I am currently employed as a Principal Research Fellow at the Queensland Institute of Medical Research (QIMR) located at 300 Herston Road, Herston, Queensland 4006, Australia. In my capacity as a Principal Research Fellow, I head the Molecular Vaccinology Laboratory at QIMR. I am concurrently employed as an Adjunct Associate Professor at Australian Center for International & Tropical Health and Nutrition and Dept Microbiology at the University of Queensland, Australia.

4.   I have extensive experience in the fields of immunology, virology, and vaccine technology. References containing examples of my work are included in my *Curriculum Vitae*, a copy of which is affixed hereto as Exhibit AS1.

5.   I understand that The Present Application was filed on May 22, 2000 as a continuation application of USSN 08/776,337 filed April 21, 1997 which is a US national phase application of International application No. PCT/AU95/00461 filed on July 27, 1995. The International application claims priority from Australian Patent Application No. PN1009 filed February 8, 1995 and from Australian Patent Application No. PM7079 filed July 27, 1994.

6.   I consider myself to have been in possession of at least ordinary skill and knowledge in the arts relevant to this invention when the invention was made (i.e. before July 27, 1994) and at all times until The Present Application was filed on May 22, 2000.

7.   I have read and understood the specification and pending claims of The Present Application and an Office Action by the United States Patent and Trademark Office mailed on May 21, 2002 in connection with The Present Application. I have also read the revised claims of The Present Application which I understand the applicants have submitted in response to the May 21, 2002 Office Action. A copy of the revised claims is affixed hereto as Exhibit AS2.

8.   I make this declaration in response to the Examiner's rejection of pending Claims 14-34 of The Present Application at paragraph 7 of the Office action mailed on May 21, 2002.

9.   I understand that the Examiner has rejected The Present Application on the ground that the only disclosed use of the claimed polynucleotide is as a vaccine, and the Examiner considers there to insufficient guidance and *in vivo* working examples provided in the specification as filed to demonstrate that a polynucleotide comprising any undisclosed CTL epitopes would be useful for preventing any undisclosed disease. The Examiner does state that the specification enables the production of a recombinant vaccine CTL polyepitope-based composition comprising a polynucleotide encoding CTL epitopes as

depicted in Figure 5 derived from pathogens MCMV, Influenza Virus, EBV, Adenovirus and EG7 tumor for use as vaccines.

10.   The Examiner also alleges in the May 21, 2002 Office Action that the specification fails to provide any enablement for vaccine compositions and their use in vaccination against any HIV. The Examiner considers that the specification as filed fails to provide guidance as to which polynucleotide encoding which CTL epitope from HIV can be used in a recombinant nucleic acid vaccine against HIV infection. The Examiner also states that the field of HIV research is unpredictable, because notwithstanding 10 years of extensive research into HIV, not a single efficacious vaccine against HIV has been produced. Accordingly, the Examiner considers that it would require an undue burden of experimentation to produce a polynucleotide encoding a polyepitope polypeptide comprising an HIV CTL epitope from the teaching provided in the specification.

11.   I strongly disagree with these allegations by the Examiner. My reasons for refuting the Examiner's position are detailed in the following paragraphs.

12.   The Present Application provides constructs encoding a plurality of CTL epitopes into a single fusion protein (i.e a "polyepitope" polypeptide). For expressing a fusion polypeptide, it is well known that there should not be a translation initiation codon (i.e. the codon ATG associated with a Kozac sequence) between sequences encoding each epitope, otherwise that codon might be used to initiate independent translation of each epitope. By omitting internal translation initiation sequences from the polyepitope polypeptide, we ensured that the plurality of CTL epitopes were expressed as a single polypeptide. In the context of the polynucleotide structure, this means that nucleic acid sequences encoding the CTL epitopes, and any intervening sequences if present, are presented as a single open reading frame, positioned between a single upstream promoter sequence and a single downstream transcription terminator sequence. Additionally, a single translation initiation codon and Kozac consensus sequence are placed at the commencement of the open reading frame for the plurality of CTL epitopes. These features are described, for example, at paragraph 45 of The Present Application. This "polyepitope" arrangement is distinct from the "minigene" arrangement as described by

Whitton *et al.*, *J. Virol. 67*, 348-352, 1993 wherein both CTL epitopes coding sequences were preceded by translation initiation sequences. Moreover, our approach was new because, as stated at paragraph 3, lines 7-8, of The Present Application, the "influence of sequences flanking CTL epitopes on the proteolytic processing of these epitopes remains controversial". Thus, it was not known or suggested before the invention how to produce a single polypeptide vaccine comprising multiple CTL epitopes, where each epitope is individually immunogenic.

13. First, since the Examiner appears to be treating all claims of The Present Application as vaccine claims, I would like to bring to the attention of the Examiner the fact that the claimed polynucleotide has additional utility to its use as a vaccine in the prevention of particular diseases.

14. As a skilled immunologist, I was well aware in 1994 and at the filing date that a CTL epitope had utilities extending beyond its use as a vaccine. For example, the polyepitope-encoding constructs of the present invention are also useful in the study of cellular and immune responses, or for the *in vitro* stimulation of CTLs (e.g. for adoptive transfer), in addition to their utility as components of protective vaccines. Additionally, epitopes and the polyepitope constructs can be used in diagnostic applications.

15. I also note the following U.S. patents filed before 1994 that describe CTL epitopes of HIV-1 and for which claims were allowed to uses other than vaccines.

(i) U.S. Patent No 5,700,635 to McMichael *et al.* (Exhibit AS3) describes a CTL epitope from the HIV-1 *gag* protein and its use in the detection of HIV-1 gag-specific CTLs. The allowed claims are directed to peptides comprising the amino acid sequence of the CTL epitope, compositions comprising same and to methods for assaying for the presence of HIV-1 specific CTLs;

(ii) U.S. Patent No 5,932,218 to Berzofsky *et al.* (Exhibit AS4) describes a CTL epitope from the HIV-1 *env* protein and its use as an immunogen to generate broad immune responses in a host, to assess immune responses in virus-infected hosts and as a diagnostic reagent to detect viral infection. The allowed claims are

directed to *both* polypeptide immunogens and methods of eliciting CTL responses and high titer neutralizing antibodies; and

(iii) U.S. Patent No 6,294,322 to Berzofsky *et al.* (Exhibit AS5) describes a CTL epitope from the HIV-1 gp160 protein and its use in generating high titer neutralizing antibodies and enhancing CTL responses. The allowed claims are directed to *both* polypeptide immunogens and methods of diagnosing exposure to HIV-1.

16. The patent documents referred to in the preceding paragraph demonstrate clearly that compositions comprising CTL epitopes were known before the present invention to have broader utility than as vaccines. In the present case, the fact that each CTL epitope in the polyepitope composition is correctly processed and presented by APCs makes the claimed constructs useful for detecting a plurality of CTL responses, or in eliciting simultaneous CTL responses to a plurality of different CTL epitopes.

17. The patent documents referred to at paragraph 15 indicate clearly that the US Patent Office practice does not equate claims directed to peptides comprising CTL epitopes with claims directed to vaccines. Proceeding on this basis, I disagree strongly with the position taken by the Examiner in the May 21, 2002 Office Action. At the very least, the present Examiner should provide The Present Application equitable consideration in respect of the polynucleotide claims.

18. Second, I strongly disagree with the Examiner that the specification discloses only a polynucleotide comprising multiple (up to ten) murine CTL epitopes as depicted in Figure 5 from the pathogens listed in Table 2.

19. Example 1 of the specification as originally filed taught a construct encoding a polyepitope consisting of nine HLA-restricted (i.e. human) EBV CTL epitopes as listed in Table 1 of the specification. The nine CTL epitopes were flanked by two B cell epitopes. The polynucleotide encoding a single polypeptide consisting of the nine CTL epitopes and two B cell epitopes incorporated a single Kozac consensus sequence and a single translation start site upstream of the open reading frame encoding the epitopes (i.e.

there were no internal translation initiation sequences between the sequences encoding the CTL epitopes). The minimal CTL epitopes were each only 9-10 amino acids in length and, as a consequence, the CTL epitopes were substantially free of nucleic acid sequences encoding naturally occurring flanking sequences (*see* final two lines of paragraph 36 and the description of Table 1). The construct was cloned into vaccinia virus and used to infect target cells, which expressed the HLA alleles restricting each epitope. HLA matched human CTL clones specific for each epitope were then used as effector cells. The positive results presented in Figures 2 and 3 of the specification indicated that each epitope in the expressed human polyepitope was processed within the human target cells and recognized specifically by human CTL clones from EBV-infected human patients. In the absence of conducting human clinical trials, this approach is well recognized for assessing stimulation of human T cells by a vaccine composition and would be expected in the field to indicate efficacy.

20. Our general approach to producing the claimed constructs is independent of the precise sequence of any CTL epitope included in the polyepitope polypeptide and, as a consequence, is not limited in application to a particular CTL-encoding nucleic acid sequence. The described method is generic rather than being limited to the exemplified polyepitope polypeptides or even specific CTL epitopes because, if the minimal CTL epitopes are known, then the process for selecting a subset of epitopes is mechanical based upon their HLA restriction and their predicted ability to protect against disease. The formulation of individual epitopes into a single polyepitope can be readily achieved following the teaching in The Present Application without undue experimentation. For example, following the teaching provided in The Present Specification, a skilled person seeking to produce an HIV vaccine would select from the literature a range of minimal CTL epitopes from appropriate HIV proteins (e.g. *gag*, *env*, *nef*, etc) where multiple epitopes from each protein may be chosen to cover a range of HLA restrictions (e.g. A2, B8, A24, B35, etc.).

21. The selection of specific CTL epitopes was not a major obstacle to performing the invention presently claimed, even in 1994. Before 1994, the general structure of a CTL epitope had been elucidated as a peptide of about 7-10 amino acids in length which is

incorporated within the groove in the major histocompatability complex type 1 (MHC-1) surface antigen on antigen presenting cells (APC) and then "presented" to CD8+ T cells, otherwise known as cytotoxic T lymphocytes (CTL) (*see* Rammensee *et al., Ann. Rev Immunol. 11,* 213-244, 1993; Exhibit AS6).

22.   Whilst the principles of CTL recognition were initially established in murine systems, subsequent experience demonstrated that those principles also applied to human cells (e.g., Townsend *et al., Cell 44,* 959-968, 1986; Exhibit AS7). Townsend *et al.* provided the first definition of the concept that CTL recognised short peptide fragments and that both murine and human CTLs recognize degraded peptide fragments of proteins in association with MHC Class I molecules. The difference lies only in the detailed selection of the CTL epitopes and not in the mechanism by which the immune response recognizes a particular epitope. The concept of the general mechanistic similarity between murine and human CTL presentation is also referred to by Rammensee *et al* 1993 (Exhibit AS6). Accordingly, the protective responses of example 2 acknowledged by the Examiner in the May 21, 2002 Office Action, also indicate efficacy of the invention for humans.

23.   Before the filing date of The Present Application, Rammensee *et al., Ann. Rev Immunol. 11,* 213-244, 1993 (Exhibit AS6) also describe a total of 58 minimal mouse and human CTL epitopes, including CTL epitopes from HIV-1 reverse transcriptase, HIV-1 gag, and HIV-1 gp120 proteins. The CTL epitopes disclosed by Rammensee *et al.* represent only a fraction of CTL epitopes known at the time of the invention [*e.g.,* Khanna *et al., J. Exp. Med 176,* 169-176, 1992 (Exhibit AS8) described CTL epitopes from EBV; U.S. Patent No 5,700,635 to McMichael *et al.* describes a CTL epitope from the HIV-1 *gag* protein (Exhibit AS3); U.S. Patent No 5,932,218 to Berzofsky *et al.* describes a CTL epitope from the HIV-1 *env* protein (Exhibit AS4); and U.S. Patent No 6,294,322 to Berzofsky *et al.* describes a CTL epitope from the HIV-1 gp160 protein (Exhibit AS5)].

24.   I also note that in the Office Action mailed on May 21, 2002, the Examiner also acknowledges several prior art CTL epitopes from a wide range of pathogens and tumor antigens, such as, for example, the following minimal CTL epitopes:

(i) a CTL epitope from CMV and a recombinant vaccine comprising same as described by Del Val *et al., J. Virol. 65,* 3641-3646, 1991;

(ii) a CTL epitope from Influenza Virus as described by Latron *et al., Proc. Natl Acad. Sci (USA) 88,* 11325-11329, 1991;

(iii) CTL epitopes from EBV as described by Burrows *et al., J. Gen. Virol. 75,* 2489-2493, 1994; and

(iv) CTL epitopes from the melanoma tumor antigens MAGE-1 and MAGE-2 and their potential use in vaccines, as described by Celis *et al., Proc. Natl Acad. Sci (USA) 91,* 2105-2109, 1994 and references cited therein at paragraph 1 of Celis *et al.*

25. The specification of The Present Application also discusses in a general sense CTL epitopes from one or more pathogens or tumor antigens, and exemplifies CTL epitopes from human EBV (Table 1) and from a range of murine pathogens as listed in Table 2, specifically Influenza Virus, Adenovirus, Sendai Virus, *Plasmodium berghei*, MCMV and LCMV.

26. Before the present invention, I was also aware of several well-established methods for determining CTL epitopes from any source. For example, predictive algorithms may provide some indication of the presence of a CTL epitope in an antigen, however such methods are in no way definitive. CTL epitopes were also known to be defined in a chromium release assay wherein the minimal amino acid sequence capable of making a target cell susceptible to CTL (usually CD8+) lysis was determined. Generally, target cells were labelled with radioactive chromium, sensitised with each of the series of truncated synthetic peptides, and mixed with activated T cells, and the extent of lysis was measured by the release of soluble chromium. This methodology precedes 1994 and is accepted by all immunologists. By 1997, numerous minimal CTL epitopes had been defined by chromium release assay (e.g., Rickinson and Moss, *Ann. Rev. Immunol. 15,* 405-431, 1997; Exhibit AS9). Additionally, the ELISPOT assay enumerates the $CD8^+$ T cell response to a given CTL epitope, via a measurement of interferon (IFN)-gamma

release. As well as measuring the frequency of CD8 T cells specific for defined CTL epitopes, the ELISPOT assay is used to scan peptide sequences to define new CTL epitopes. One of the early references to use of ELISPOT for analysis of epitope specific CD8 T cells was Miyahira *et al.*, *J. Immunol. Methods 181*, 45-54, 1995 (Exhibit AS10).

27.   As one skilled in the art, I can also state that the artisan would find the matter of making the constructs of the present invention, armed with the guidance provided in the specification, a routine matter. The materials and general molecular procedures for constructing the polyepitope constructs were readily available to the artisan at the time the application was filed.

28.   Based upon the disclosure in the specification and what was known before the filing date of The Present Application, I disagree with the Examiner's conclusion that there are insufficient working examples of the claimed polynucleotides to enable a skilled person to produce the full scope of polynucleotides claimed.

29.   In my opinion, there is sufficient teaching provided in The Present Specification to enable a skilled person to conjoin any number of CTL epitopes substantially free of natural flanking sequences, from any number of different antigens of a number of different pathogens. As the prior art disclosed a wealth of CTL epitopes as acknowledged by the Examiner in the May 21, 2002 Office Action and The Present Application also disclosed a large number of CTL epitopes from the antigens of human and murine pathogens and two divergent polynucleotides encoding such fusion polypeptides (Examples 1 and 2), the specification contains a sufficient teaching to enable a skilled molecular biologist or immunologist to produce such polynucleotides without any undue burden of experimentation.

30.   In support of this conclusion, I submit herewith the following publications that use the methods disclosed in The Present Application to produce new polyepitope-encoding constructs:

(i)   Gardner *et al., In: Proc. 12th World AIDS Conference, Geneva, Switzerland, June 28-July 3, 1998,* pp 295-298 (Exhibit AS11). In particular, Gardner *et al.* teach a

polyepitope construct encoding multiple tandem HIV HLA A2-restricted CTL epitopes (Table 1), its delivery by modified vaccinia virus Ankara (MVA), and the testing of same in HLA A2 transgenic mice prior to human clinical trials (p. 296 "Polytope vaccines against HIV/AIDS"). Gardner *et al.* state at page 296, lines 1-5, that the "polytope approach has now been shown to work for a variety of epitopes, diseases and vectors including HIV and Modified Vaccinia Ankara (MVA) (Hanke et al., 1998), malaria and Ty particles (Gilbert et al., 1997), different viruses and vaccinia (An and Whitton, 1997), and influenza and lipopeptides (Sauzet et al., 1995).

(ii)   Mateo *et al., J. Immunol. 163*,4058-4063, 1999 (Exhibit AS12) describe an HLA A2-restricted polyepitope vaccine against melanoma comprising ten CTL epitopes wherein vaccinated HLA A2 transgenic mice generated CTL specific for the multiple epitopes that recognized and killed human melanoma cells;

(iii)   Woodberry *et al., J. Virol 73*, 5320-5325, 1999 (Exhibit AS13) describe an HIV-1 CTL polyepitope construct encoding seven tandem HIV-1 CTL epitopes, that induces CTL specific for the multiple epitopes in transgenic HHD mice [i.e. transgenic mice having a transgene comprising the alpha-1(H) and alpha-2 (H) domains of human HLA A2 linked to the alpha-3 transmembrane and cytoplasmic domains of mouse H-2D^b (D), with the alpha-1 domain being linked to human beta-2 microglobulin), such that the only MHC molecule expressed by the mice is the modified HLA A2 molecule);

(iv)   Firat *et al., Eur. J. Immunol. 31*, 3064-3074, 2001 (Exhibit AS14) describe a polyepitope construct encoding thirteen HIV-1 CTL epitopes and its use to induce long-lasting CTL responses against the epitopes in HHD mice; and

(v)   Suhrbier *Expert Rev. Vaccines 1*, 207-213, 2002 (Exhibit AS15) which reviews the polyepitope vaccines shown to induce multiple protective CD8 T cell responses (i.e. CTL responses) in murine, monkey, and *in vitro* systems (*see* Table 1) and those polyepitope vaccines presently in, or near to, human clinical trials (*see* Table 2).

In particular, Suhrbier (2002) (Exhibit AS15) summarizes the efficacy of such polytope constructs at page 208, left column, as follows:

> "Perhaps surprisingly, every individual epitope within such conjoined strings of epitopes *has been shown repeatedly to be immunogenic.*" *(emphasis added)*

31.   As for vaccines, at least one construct exemplified in The Present Application (i.e. Example 2) did confer a protective immune response. As noted *supra*, Example 2 of the specification as originally filed taught that the CTL responses induced by the polyepitope were, in fact, protective.   This analysis was performed using a tumor model (i.e. ovalbumin-transfected EL4 cells designated "EG7 tumor cells" which are innoculated into C57BL/6 mice), and Balb/c mice infected with MCMV (paragraph 56).

32.   Notwithstanding that example 2 is the only *in vivo* working example provided in the specification of a protective immune response, the example provided included CTL epitopes to a wide range of different pathogens, indicating that the pathogen *per se* was not a limiting factor in producing a protective immune response.   Indeed, this technology is broadly applicable to any vaccination situation in which the generation of CTL response is important for providing protective immunity.

33.   Affixed hereto is a copy of a review article I wrote in 1997 (Suhrbier, *Immunol. Cell. Biol. 75*, 402-408, 1997; Exhibit AS16) which demonstrates the well-established fact that CTLs are required for protection against many diseases and that effective vaccines against many diseases require induction of specific CTL responses.   For example, Suhrbier states at page 404, left column, paragraph 3, as follows:

> "CTL provide immune control of EBV-infected B cells. A vaccine capable of inducing EBV-specific CTL is therefore likely to protect against both infectious mononucleosis (IM) and post-transplant lymphoproliferative disease (PTLD); diseases which are characterised by uncontrolled expansion of EBV-infected B cells....considerations make a compelling case for the epitope-based approach for an EBV CTL vaccine...a small number of EBV-specific CTL were sufficient to clear an X-ray visible PTLD."

Suhrbier also states, at page 404, bridging left and right columns:

> "Cytotoxic T lymphocyte epitopes delivered by DNA vaccination have given therapeutic and prophylactic protection against tumours in animal models, as have CTL epitopes delivered by vaccinia..."
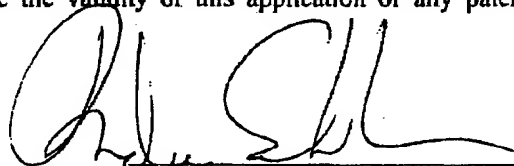
Suhrbier also states at page 405, left column, as follows:

> "There is now a considerable body of compelling indirect evidence that CTL have a role in preventing or limiting (or even clearing): (i) initial HIV infection; and (ii) progression to AIDS....many observations still provide convincing evidence for the protective role of CTL in HIV/AIDS."

34. It is well accepted in the immunological arts to use systems other than a phase II human clinical trial to provide evidence for the efficacy of a vaccine formulation. Murine vaccination studies and *in vitro* assays using T cells from human also provide evidence of vaccine efficacy.

35. That CD8 T cells or CTL likely provide protection against certain human diseases can be established using mouse models and *in vitro* studies of T cells derived from infected humans. That a vaccine modality is capable of inducing CTL immunity can similarly be established using murine studies and *in vitro* studies of human T cells.

36. For example, to assess the efficacy of HLA A2 restricted CTL epitope-based vaccines, HHD mice can be used. Alternatively A2/K^b mice can also be used for testing CTL response to vaccine compositions comprising HLA A2-restricted CTL epitopes (Shirai *et al, J. Immunol 154*, 2733-2742, 1995).

37. Alternatively, the human T cells can be stimulated *in vitro* with the polyepitope or an individual CTL epitope thereof and T cell activation measured using cytotoxicity assays or ELISPOT. For example, Smith *et al, Clin Cancer Res 7*, 4253-4261, 2001, used a polyepitope to induce human CTL responses *in vitro*. Additionally, Thomson *et al., Proc. Natl. Acad. Sci USA 92*, 5845-5849, 1995, showed that the EBV polyepitope was able to restimulate CTL s from EBV-infected individuals.

38. Accordingly, the Examiner is in error in requiring data on protection as evidence that the specification enables the production of a vaccine composition. In any event, I consider that the protective data provided in example 2 of The Present Application clearly enables a skilled immunologist to produce a DNA vaccine encoding a polyepitope polypeptide of the invention comprising any number of different CTL epitopes. The Present Application makes it possible to formulate a vaccine (either DNA or polypeptide) containing sufficient antigenic content to induce CTL responses to one or more proteins within a pathogen or a tumour over a broad cross-section of the human population. The incorporation of nucleic acid encoding CTL epitopes into a polyepitope construct for use as a vaccine would be routine in view of the skill in the arts and the guidance provided by the specification.

39. In summary, the specification as filed provided a sufficient teaching to enable a skilled immunologist to produce a polynucleotide comprising a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) epitopes wherein each CTL epitope is substantially free of sequences encoding peptide sequences naturally found to flank that CTL epitope and wherein at least two of the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence that does not comprise a translation start methionine, irrespective of the precise structure or number of the CTL epitopes employed, the nature of the pathogen from which the CTL epitopes were derived, or the vector system employed. I also consider that the specification enables such a person to produce and test nucleic acid vaccines comprising the subject constructs.

40. I declare that all statements made of my knowledge are true and all statements made on the information are believed to be true; and, further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereupon.

Date: 20/11/02

ANDREAS SUHRBIER

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Suhrbier, et al.

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

DECLARATION OF ANDREAS SUHRBIER

EXHIBIT AS1: *Curriculum vitae* **of Andreas SUHRBIER**

CURRICULUM VITAE

SUMMARY. Andreas Suhrbier is a principal research fellow with QIMR and holds conjoint associate professor positions with the Australian Centre for International Tropical Health & Nutrition, and the Dept Micro & Parasit., University of Queensland. He has a total of 69 accepted publications including 1 Nature Medicine, 1 PNAS, 2 J. Exp. Med., 6 J Immunol., 7 J Virol., and seven invited reviews. He has been a CI on three NIH (USA) grants. Major scientific contributions include setting up the first phase I CTL-based Epstein-Barr virus vaccine trial, discovery and development of the polyepitope or polytope approach for delivering multiple cytotoxic T cell epitopes, directly demonstrating for the first time that CTL alone can protect against retroviral infection, and discovery of the first arbovirus of marine mammals.

Fully funded international trips in the last 3 years include invitations to the Harvard Medical School (Boston), Lyon (Aventis, France), California-Aust. Biotech Partnering Conf. (San Diego), Elmau Workshop on MVA (Germany) and Institute Pasteur (Paris). He is also an invited speaker at the World Congress on Vaccines and Immunization, 2002 (Croatia). Recent funded local invited speakerships include the Australian Virology Group 2001, Hanson Symposium 2000, 8th Arbovirus Conf. 2000, The Aust. Soc. Infect. Dis./Aust. Coll. Trop. Med. meeting 1999, and CTTAC/ANCARD HIV-vaccine workshop 1999.

He has been interviewed/involved in various media activities including Qantum, Norman Swan health report, New Scientist, national science week panel member, ABC science in the pub panel member and several local radio and newspaper reports.

He has been an inventor on 11 patents, 6 of which have been commercialised (sold) to international and Australian pharmaceutical companies. Currently he is a consultant for CSL Ltd., Peplin Biotech (Australia), Bavarian Nordic (Germany), and last year he consulted for Aventis Pasteur (France).

NAME   ANDREAS SUHRBIER

DATE OF BIRTH   2nd May 1960

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EMPLOYMENT HISTORY

2000 - PRESENT   QIMR.
                        Principal Research Fellow (PRF); Molecular Vaccinology
                        Laboratory, Division of Infectious Diseases and Immunology.

                        Adjunct Associate Professor; Australian Center for
                        International & Tropical Health & Nutrition, and Dept.
                        Microbiology, University of Queensland.

1995 - 2000   QIMR. Lab head of Molecular Vaccinology.

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Senior Research Fellow (SRF); Epstein-Barr virus Unit (SRF1,
5/12/95).

Adjunct Senior Lecturer; Australian Centre for International &
Health & Nutrition, and Dept. Microbiology, University of
Tropical Queensland.

1992 -1995
QIMR.
Research Fellow; Epstein-Barr virus Unit

Adjunct Lecturer; Australian Centre for International &
Tropical Health & Nutrition, University of Queensland.

1994-1999.
Program Leader of program "Induction of cytotoxic T cells"
Co-operative Research Center for Vaccine Technology.
Member of the Scientific Advisory Committee (and Research
and
Development Advisory Committee till 1996).

1990 - 1992.
QIMR. Senior Research Officer/Wellcome Fellowship/Royal
Society
Fellowship. Malaria & Arbovirus Unit.

1986 - 1989.
Research Officer,
Imperial College of Science and Technology,
Department of Pure & Applied Biology, ,
Molecular and Cellular Parasitology Group,
Prince Consort Road, London, SW7 2BB.

MAJOR SCIENTIFIC CONTRIBUTIONS:

- Discovered the first arbovirus of marine mammals, which also emerges to be the first
arbovirus transmitted by lice and the first alphavirus of Antarctica. The story was widely
publicized and appeared in New Scientist.

- Directly demonstrated for the first time that CTL alone can protect against the establishment
of a latent retroviral infection using an ovine retrovirus model. The work was published in
Nature Medicine.

- Development of the polyepitope or polytope approach for delivering multiple cytotoxic T
cell (CTL) epitopes. The approach is patented by the CRC-VT and has been licensed to
Bavarian Nordic GmbH, and CSL Ltd. for EBV, HIV and melanoma.

- Setting up the first phase I CTL based Epstein-Barr virus (EBV) vaccine trial. A critical
step was award of National Institutes of Health, USA grant (US$160,000 p.a. for 4 years).
The CRC-VT has filed several patents from this work and is developing an EBV vaccine in
partnership with CSL Ltd.

- Establishment of the first *in vitro* model of Ross River virus disease (RRVD), which indicated that persistent virus in synovial macrophages is the cause of RRVD, a disease afflicting up to 7000 Australian annually. Also the first group to complete a prospective survey of RRVD using validated quality of life questionnaires.

- Characterisation of the second known liver stage specific antigen of malaria and manufacture of the first monoclonal antibody specific for a liver stage antigen. Extensive immunohistochemical data also illustrated how unrealistic the murine models of malaria are for understanding CTL immunity to liver stage parasites.

- Achieving for the first time the complete development *in vitro* of the vertebrate cycle of malaria (*P. berghei).*

PUBLICATIONS (*indicates key papers, [IN]indicates invited papers, #book chapters).

70. G. A. Darnell, T. M. Antalis, R. W. Johnstone, B. W. Stringer, S. M. Ogbourne, D. Harrich, and **A. Suhrbier**. Plasminogen Activator Inhibitor-2 has an Intracellular Activity as a Retinoblastoma Binding Protein (submitted).

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GRANTS
Current

Australian Antarctic Division 2002 Isolation and characterization of arboviruses in seals and birds Slade and Suhrbier AU$10,000

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NH+MRC. 1997-2003. The immunopathology of epidemic polyarthritis. **A. Suhrbier**. A$ 58,000 p.a.

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Past support

ANCARD. 1998-2001. A cytotoxic T cell based vaccine for HIV. A. Suhrbier and S. Elliott A$90,000 pa.

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NH+MRC; The use and effects of perforin independent human cytotoxic T cell lytic mediators. 1996-8. **A. Suhrbier.** (A$ 48,000 p.a.).

CRC for vaccine technology; T cell epitope identification. 1995-7. **A. Suhrbier,** B. Coupar, M. Andrews, R. Khanna, D. Jackson and D.J. Moss. (A$ 73,230pa)

CRC for vaccine technology; 1995-7. CD8 and CD4 T cell polyepitope vaccines: evaluation in murine systems. **A. Suhrbier,** R. Khanna, S. Elliott, B. Coupar (A$ 74,500pa)

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Sylvia and Charles Viertel Charitable Foundation. 1993-4. Ross River Virus infection; new initiatives for treatment. **A. Suhrbier** (A$20,000).

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University of Queensland New Staff Research Grant. 1993-1994. Isolation and Characterisation of Ross River virus specific T cells. **A. Suhrbier**, J. Aaskov and D.J. Moss. (A$20,000).

CRC for vaccine technology; T cell epitope processing. 1993. **A. Suhrbier**, B. Coupar, M. Andrews, R. Khanna, D. Jackson and D.J. Moss. (A$ 7,500).

UK grants;
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Leverhume Trust, UK. 1988-1989. The liver stage of malaria, a novel target for anti-malarial vaccines. (Authors; A.Suhrbier, L. Winger and R.E.Sinden).

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Fellowships;
Wellcome Trust Advanced Training Fellowship in Tropical Medicine. 1990-1993. Identification of T cell epitopes in vaccine candidates. UK pounds 30,000 pa.

Royal Society Fellowship. 1989-1990. Identification of T cell epitopes in vaccine candidates. UK pounds 2000.

R+D CONTRACT RESEARCH

Aventis Pasteur 2000. Cryopreservation DEN052. AU$210,000

Peplin Ltd. 2000 and 2001. Anti-cancer compounds. AU$ 600,000 pa.

CSL Ltd. 2001-2002. Testing ISCOM formulations. AU$ 120,000.

CSL Ltd. 1999-2000. Testing ESO-ISCOM formulations. AU$ 60,000.

CSL Ltd./ CRC VT; 1999-2001. Defining important aspects in relation to developing a vaccine to EBV. D.J. Moss, A. Suhrbier, M. Bharadwaj. AU$ 155,000 pa.

CSL Ltd./ CRC VT; 1999-2001. Development of an ISCOM-polytope vaccine. A. Suhrbier, D. Drane, J. Cox, M. Pearse. AU$ 66,000 pa.

Bavarian Nordic GmbH, Germany. 1999. Poxvirus Potency. AU$15,000.

Bavarian Nordic GmbH, Germany. 1999. Poxvirus Prime/Boost. AU$30,000.

Bavarian Nordic GmbH, Germany. 1999. Poxvirus Challenge. AU$30,000.

CONSULTANCIES

1998 - 2000.	Bavarian Nordic. Germany
1998- present	CSL Ltd., Australia
2000/2001	Aventis Pasteur, France
1999-present	Peplin Biotech, Australia

PATENTS

11. PR9093. 2001-11-27. Flavivirus Vaccine System. Inventors: A.A. Khromykh, **A. Suhrbier**. Applicant; Queensland Department of Health. Commercialised; NO.

10. PCT/AU01/01635. 2000-12-19. Rb binding protein. Inventors; Antalis T, Darnell G, Johnson, R, **Suhrbier A.** Applicant; QIMR. Commercialised; NO.

9. PCT/AU01/00679; 2001-6-7. Therapeutic agents - I. Inventors: Aylward JH, Parsons PG, Suhrbier A and Turner KA. Applicant; Peplin Biotech Ltd. Commercialised; **YES.**

8. PCT/AU01/00680; 2001-6-7. "Therapeutic agents - II. Aylward JH, Parsons PG, Suhrbier A and Turner KA. Applicant; Peplin Biotech Ltd. Commercialised; **YES.**

7. PCT/AU01/00678; 2001-6-7. "Therapeutic agents - III. Aylward JH, Parsons PG, Suhrbier A and Turner KA. Applicant; Peplin Biotech Ltd. Commercialised; **YES.**

6. WO0123577. 2001-04-05. Hybrib or chimeric polynucleotides, proteins, and compositions comprising hepatitis b virus sequences. Inventors; Langlade-Demoyen P, Michel ML, Firat H, Lemonnier F, Suhrbier A. Applicant; Institute Pasteur, Paris, France. Commercialised; NO

5. WO0048630. 2000-08-24. Immunogenic Complexes and methods Relating Thereto II. Inventors; Drane D, Cox J, Suhrbier A. Applicant; CSL Ltd.
Commercialised; **YES**

4. WO9603144/ EP0769963. 08/02/1996. POLYEPITOPE VACCINES. Inventors; **A. Suhrbier**, S. Thomson, R. Khanna, S. R. Burrows, B. Coupar, D. J. Moss. Applicant CRC for Vaccine Technology.
Commercialised - **YES**

3. WO9524925/ US5869453. 09/02/1999. CYTOTOXIC T-CELL EPITOPES. Inventors; D. J. Moss, S. Burrows, R. Khanna, B Kerr, **A. Suhrbier**. Applicant; CRC for Vaccine Technology.
Commercialised - **YES**

2. WO09524926A1. 09/21/1995. FORMULATION FOR USE IN INDUCING CTL. Inventors; J. Cox, **A. Suhrbier**, S. L. Elliott. Applicant; CRC for Vaccine Technology.
Commercialised – Dropped.

1. WO09205193A1. 04/02/1992. EP00549662A1. 07/07/1993. PLASMODIUM LIVER STAGE ANTIGENS. Inventors; R. E. Sinden, **A. Suhrbier** and L. Winger. Applicant; Imperial College, London. UK. Commercialised – Dropped.

FUNDED INVITATIONS

International

June. 2002.	*Invited speaker,* Epitope-based cytotoxic T lymphocyte vaccines. Third world congress on vaccines and immunization. Opatija, Adriatic Riviera, Croatia.
Oct. 2000.	*Guest lecturer,* Anti-retroviral vaccines. AIDS Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, USA.
Oct. 2000.	*Commercial collaboration visit and lecture,* Polytope vaccines. Aventis Pasteur, Immunology Department, Marcy L'Etoile, France.
May. 1999.	*Invited participant,* California-Australia Biotech Partnering & Finance conference. San Diego, USA.
Jan. 1999.	*Invited participant,* The MVA in vaccination and immunotherapy. Elmau Workshop, Bavaria, Germany.

April. 1998.	*Commercial co-develoment meeting.* Bavaria Nordic Research Institute, Munich, Germany.
March. 1993+94.	*Invited participant (Stipend)*: National Cancer Institute and National Institute of Allergy and Infectious Diseases Discussion Group. New York, USA.

Australian

Dec, 2001	*Selected speaker*: The natural history of Ross River virus induced epidemic polyarthritis. Qld Health & Medical Sciences Meeting, Brisbane, Qld.
Dec, 2001	*Invited plenary speaker*: Viruses, vaccines and CTL. Australian Virology Group, Fraser Island, Qld.
Nov, 2001	*Invited speaker*: Health, nonsense and money. The Australian Skeptics National Conference, Brisbane.
May, 2001	*Invited speaker*: Anti-cancer vaccine and drug strategies. Peter MacCallum Cancer Institute, Melbourne.
Nov, 2000	*Invited speaker*: Anti-cancer vaccine strategies. Hanson Symposium. Adelaide.
July, 2000	*Invited speaker and session organiser*: " General practioner based survey of Ross River virus induced epidemic polyarthritis " 8th Arbovirus Research in Australia Conference, Couran Cove Resort, Stradbroke Island, Qld., 3-7th July 2000. (Session on Ross River virus and clinical disease).
April, 1999	*Invited speaker*: "Ross River virus" The Australasian Society for infectious diseases & The Australasian College of Tropical Medicine Annual Scientific Meeting. Cairns.
March, 1999	*Invited speaker*: "HIV polytope vaccines" CTTAC/ANCARD Australian prophylactic HIV-vaccine research workshop. Sydney.
December, 1998	*Invited speaker*: "EBV and polytope vaccines" and "Polytope vaccines for melanoma". *Invited chair*: Immune responses to viral infections. Australian Society for Immunology, Melbourne.
September, 1997	*Invited speaker*: The immunopathogenisis of Ross River virus disease. Australian Society for Microbiology Scientific Meeting, Adelaide.

May, 1997	*Invited speaker*: The immunopathogenisis of epidemic polyarthritis. Australian Rheumatology Association 40th annual scientific conference. Brisbane, Qld.
November, 1996	*Invited chair*: Flaviviruses. Arbovirus Research in Australia, 7th Symposium. Gold Coast, Qld.
November, 1996	*Invited chair*: Advances in Vaccine Technology. ASMR 35th Nat. Sci. Conf.. Gold Coast, Qld.
June, 1996	*Invited speaker*: Developments in Clinical aspects of Ross River virus and vaccine prevention. Sunshine Coast Regional Health Authority. Redcliffe Hospital, Brisbane.
April, 1996	*Invited speaker*: Immunopathology of epidemic polyarthritis. The Australian Society for Microbiology. Serology/Virology special interest group. Qld. Health, Brisbane.
September, 1995	*Invited speaker*: Implementation of an EBV vaccine trial. Australian Tropical Health & Nutrition Conference. Brisbane, Australia.
March, 1995	*Invited speaker*. Vaccine strategies for the induction of CD8+ cytotoxic T cells. pIII. Vaccines 95, Lorne, Vic., Australia.
September 1992.	*Invited speaker*: Peptide epitope induced apoptosis of human cytotoxic T lymphocytes. Cell and Molecular Biology of Apoptosis. QIMR.

UNDERGRADUATE TEACHING

June 2000	"The PhD Experience". Brisbane Postgraduate Medical Research Student Conference 2000. . Brisbane Convention Centre, Brisbane, Qld..
Oct 1999	"Advances in antiviral strategies". 3rd year Immunol/Micro. Uni. Adelaide.
Oct 1999	"Bioterrorism debate". Q fever conference. QIMR..
1996 & 1997	Dept Microbiol. Uni. Qld. Advanced Immunology MY 352 and Immunology BL201. (Total 8 lectures per annum).

POSTGRADUATE TEACHING (not including regular QIMR, CRC and EBV unit seminars).

Invited speaker "Vaccines for rich and poor" *In* Challenges of Tropical Public Health, series. ACITHN for masters/diploma students. April 2000.

Invited speaker: "Dancing with T cells". Postgraduate seminar series. Uni. Adelaide. 11th Oct 1999.

Invited speaker: "CTL Immunotherapy". Postgraduate seminar series. Austin Repat. Hosp. Heidelburg. 21st July 1999.

Invited reviewer: Immunology projects workshop. Uni. Dept. Med., QE II Med. School, Perth,. Aug. 1998.

Invited reviewer: RRV projects workshop. UQ Dept. Med., Townsville, Mar. 1997.

Faculty member: ASI postgraduate teaching course in immunology. Gold Coast. Dec 1995.

Invited speaker: "Delivery of determinants". CRC for Vaccine Technology and the Immunology Group of Victoria 1995 seminar series. WEHI, Melbourne. June 1995.

Invited speaker: "Antigen processing and presentation". CRC for Vaccine Technology seminar series, Brisbane, Qld. May 1995.

Invited speaker: "Role of CD8+ cytotoxic T cells in transplantation". Transplantation Society of Australia and New Zealand postgraduate course, Brisbane. March 1995.

Invited speaker and faculty member (Paid): "Cytotoxic T cell vaccines". Australian Society for immunology/FIMSA/AMRAD. Advanced training course in Immunology. Twin Waters, Maroochydore. Qld. Australia. October 1994 .

Student supervision;

Principal supervisor;
- Grant Darnell, PhD. PAI-2 intracellular function. 2001-
- Itaru Anraku, PhD. Kunjin replicon vaccine vectors. 2001-
- T. Woodberry. PhD. Development of an mucosal polytope vaccine for HIV. 1998-2002
- L. Mateo. MMedSci. Role of CTL in retroviral infections. 1998-2000.
- Dr May La Linn. PhD. Immunopathogenesis of Ross River virus induced epidemic polyarthritis. 1995-1999.
- Scot Thomson. Processing of T cell epitopes. Part time, 1993-1997.

Associate supervisor, PhD;
- Andrew Hislop. Mechanisms of immunity &development of vaccines against retroviral induced disease. Full Time. 1997-1999.

COMMITTEES

Peplin Biotech Ltd;
2000 - present   Medical Advisory Panel

NH+MRC;
2000/02   Invited attendant JDFI-NHMRC meeting on Type I diabetes
vaccines.

QIMR Committees;
  1999 - present   QIMR intellectual property committee.
  1994 - present   QIMR higher degrees committee.

Co-operative Research Centre for Vaccine Technology/CSL Ltd.;
  1994-1999.   Program Leader of "Induction of cytotoxic T cells" program.
  1994-1996   Scientific Advisory, and Research & Development Advisory
Committees.
  1999-2001   Key scientist's committee.

  1997-2001   EBV vaccine project management team.
  1998-2001   Project management leader. ISCOM polytope.

  2001-present   Project management leader. Targeting particulate immunogens.

GRANT REVIEWING

  - NH+MRC since 1995.
  - Qld/NSW/VIC cancer funds since 1997. On QCF review committee 1998.
  - Princess Alexandra Hospital Foundation, 1999-

MEDIA ACTIVITY

  23rd Aug 2002   CRC-VT Science in the Pub panel member "Science of
                         Medicine: Truth Bollocks and Quackery" Brisbane, Qld. ABC
                         radio interview promo.

  1st June 2002   ABC Science in the Pub panel member "Could we immunise
                         against AIDS, cocaine or chocolate?"Mount Isa, Qld.

  12th May 2001   National Science week. ABC radio panel on vaccination. Talk
                         on complementary medicines and on discussion panel on
                         "Science one step ahead of disease?"

  12th May 2001   New Scientist. "Out of the Blue". p. 6. Article about the newly
                         discovered arbovirus of seals. Was followed by radio interviews
                         on local ABC radio, Triple J, Radio Newcastle, Radio Adelaide,
                         The Drive Show etc

  29th Jan 2001   Normal Swan, Radio National Health Report, interview on
                         licensing of the Polytope patent to Bavarian Nordic GmbH for

the development of an HIV vaccine. Richard Aedy section on vaccines.

July 1999.	Article in Australasian Science, pp 25-27. New Approaches to HIV Vaccines. A Suhrbier & T Woodberry.
28th April 1998	ABC Science in the Pub panel member "Can we live to 150?" http://newt.phys.unsw.edu.au/~mgb/SciPub/scipub17.html Went to air Feb 2000.
7th Oct 1998	Normal Swan, Health Report, interview on BLV vaccine published in Nature Medicine. http://abc.net.au/rn/talks/8.30/helthrpt/stories/s13221.htm
Mar 1996.	ABC Qantum program on the glandular fever vaccine. http://abc.net.au/quantum/info/q96-30-3.htm

MOLECULAR VACCINOLOGY LABORATORY AWARDS AND PRIZES

March, 2002	-Grant Darnell. Trainee travel awards to attend the 3rd International Serpin Meeting, Chicago, June.2002. -QCF travel award.
June, 2001	T Woodberry, Keystone scholarship. Prime boost strategies to enhance CTL responses depends on focus not avidity. T Woodberry, J Gardner, SL Elliott, A Suhrbier. Keystone, AIDS vaccines in the new millennium symposium, 2001, USA.
Dec, 2000	T Woodberry, QIMR Postgraduate student seminar prize and ACITHN best 1999 student publication award.
June, 2000	T Woodberry, Queensland delegate to Queens Trust Young Leaders Forum 2000.
June 1999	T Woodberry, ASI Travel Bursary Award.
July 1999	May La Linn, ACITHN best student research publication award; 2nd place.
Oct 1998.	RBH Health Care Symposium, 21-27 Oct. RBH Brisbane. M. Bharadwaj. 2nd; research presentation award, Medical & Clinical category. "Phase I human clinical trial of an EBV vaccine.
Nov/ Dec 1998.	ASI 1998 conference poster prize; Woodberry T, Gardner J, Mateo L, Eisen D, Ramshaw IA, Ffrench RA, Elliott SL,

Suhrbier A. "Polytope Vaccine Strategy for the co-delivery of multiple HIV CD8+ cytotoxic T cell epitopes." Annual meeting of the Australian Society for Immunology, Melbourne.

May 1998   .   ScienceNOW! fresh science selection winner (http://www.byc.com.au/sciencenow/) PhD student T Woodberry (http://www.byc.com.au/snmedia/tonia.htm). "New vaccine concept applied to HIV vaccine" presentation, Melbourne.

1991   Beckman Science Award, A. Suhrbier. (Based on publication record of junior QIMR researchers).

EDUCATION

1982-1985.   PhD received 31.1.86.
Title; The conservation and formation of desmosomes.
PhD Supervisor; Dr. D.R. Garrod.
CRC Medical Oncology Unit, Southampton General Hospital, Southampton, Hants.

1978-1982   BA (MA, honorary £5) Biochemistry, 2nd class honors.
New College, Oxford. (Open Scholar).

LANGUAGES SPOKEN   English and German.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Suhrbier, et al.

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

DECLARATION OF ANDREAS SUHRBIER

EXHIBIT AS2: Amended claims

14.   A polynucleotide comprising a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) epitopes wherein each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope and wherein at least two of the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence that does not comprise a methionine.

15.   The polynucleotide of claim 14, wherein the CTL epitopes are contiguous.

16.   The polynucleotide of claim 14, wherein said polynucleotide encodes at least three CTL epitopes.

17.   The polynucleotide of claim 14, wherein said polynucleotide encodes four CTL epitopes.

18.   The polynucleotide of claim 14, wherein said polynucleotide encodes nine CTL epitopes.

19.   The polynucleotide of claim 14, wherein said polynucleotide encodes ten CTL epitopes.

20.   A vector comprising the polynucleotide of claim 14.

21.   The vector of claim 20, wherein said vector is selected from the group consisting of a viral vector and a virus-like particle (VLP).

22.   The vector of claim 21, wherein said viral vector is a vaccinia vector.

23.   The vector of claim 21, wherein said viral vector is an avipox virus vector.

24.   The vector of claim 21, wherein said vector is a VLP.

25.   The polynucleotide of claim 14, wherein at least one of said CTL epitopes is derived from a pathogen.

26.   The polynucleotide of claim 14, wherein said polynucleotide comprises a nucleic acid sequence encoding CTL epitopes derived from a plurality of pathogens.

27.   The polynucleotide of claim 25, wherein said pathogen is selected from the group consisting of Epstein Barr Virus, Influenza Virus, Cytomegalovirus, Adenovirus and HIV.

28.   The polynucleotide of claim 14, wherein at least one of said epitopes is derived from a tumor protein.

29.   The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a T helper cell epitope, a B cell epitope, or a toxin.

30.   The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a T helper cell epitope.

31. The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a B cell epitope.

32. The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a toxin.

33. A nucleic acid vaccine comprising a polynucleotide comprising:

    (i) a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) epitopes wherein each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope and wherein at least two of the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence that does not comprise a methionine; and

    (ii) an acceptable carrier.

34. The nucleic acid vaccine of claim 33 wherein the CTL epitopes are contiguous

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Suhrbier, et al.

Serial No.: 09/576,101

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For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

DECLARATION OF ANDREAS SUHRBIER

EXHIBIT AS6:

Rammensee *et al., Ann. Rev Immunol. 11,* 213-244, 1993

25226358.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Suhrbier, et al.

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

DECLARATION OF ANDREAS SUHRBIER

EXHIBIT AS4:

U.S. Patent No 5,932,218 (Berzofsky *et al.*)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Suhrbier, et al.	Group Art Unit: 1644
Serial No.: 09/576,101	Examiner: Huynh, P. N.
Filed: May, 22 2000	Atty. Dkt. No.: FBRC:004/TMB
For: POLYEPITOPE VACCINES	

DECLARATION OF ANDREAS SUHRBIER

EXHIBIT AS3:

U.S. Patent No 5,700,635 (McMichael *et al.*)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
  Suhrbier, et al.

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

DECLARATION OF ANDREAS SUHRBIER

EXHIBIT AS5:

U.S. Patent No 6,294,322 (Berzofsky *et al.*)

25226358.1

PEPTIDES NATURALLY PRESENTED BY MHC CLASS I MOLECULES

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D-W-7400 Tübingen, Germany

Abstract

MHC class I molecules are peptide receptors of stringent specificity which however still allow millions of different ligands. This is achieved by the following specificity characteristics summarized as allele specific peptide motifs. Peptides are of defined length, depending on the class I allele (either 8 or 9 residues; exceptions have been observed). Typically, 2 of the 8 or 9 positions are anchors that can only be occupied by a single amino acid residue, or by residues with closely related side chains. Location and characteristics of anchors vary with class I alleles. The C terminus of the peptide ligands is frequently an aliphatic or charged residue. Such allele-specific class I peptide ligand motifs, known so far for H-2K^d, K^b, K^k, K^{km1}, D^b, HLA-A*0201, A*0205, and B*2705, are useful to predict natural T cell epitopes. The latter can be determined by extraction from cells recognized by the T cell of interest. It is not known how the class I ligands are produced in the cell, although speculative models exist. The peptide specificity of class I molecules and experimental evidence indicate that T cells are tolerant to only a small fraction of the expressed genomic sequences and are not tolerant to the remainder. The function of class I molecules is to present a collection of self-peptide samples at the cell surface for surveillance by T cells.

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N. Damle. Coexpression and functional cooperativity of CTLA-4 and CD28 on activated T lymphocytes, *J. Exp. Med*, in press, 1992). The second study shows that expression of B7 on murine tumor cells costimulates anti-tumor immunity mediated by CD8⁺ T cells (L. Chen, S. Ashe, W. A. Brady, I. Hellstrom, K. E. Hellstrom, J. A. Ledbetter, P. McGowan, P. S. Linsley, Costimulation of antitumor immunity by the B7 counter-receptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*, in press, 1992).

INTRODUCTION

Major histocompatibility complex (MHC) molecules are peptide receptors. Cell surface MHC class I molecules present peptides derived from cellular proteins to T cells. In the normal situation, all peptides are from normal cellular proteins, and the T cells are tolerant to these normal self-peptides. In pathological situations, for example, if a cell has been infected by a virus, foreign peptides are presented in addition to the normal self-peptides. The foreign peptides can now be recognized by T cells, which may destroy these cells. Thus, the system of peptide presentation by MHC class I molecules and the screening of these peptides by T cells provide the immune system with a measure to survey the interior of cells for expression of abnormal proteins and to destroy such aberrant cells. This system is instrumental in protecting the body against viral disease, or against certain other infectious agents with cytosolic parasitism, like Listeria or Plasmodium. In addition, certain tumor cells expressing peptides unfamiliar to T cells may also be eliminated. The purpose of this review is to discuss the nature of peptides presented by MHC class I molecules, the rules for peptide presentation, the ways the peptides might be processed from cellular proteins, and the significance of peptide presentation for immunity as well as for self-tolerance.

STRUCTURE OF CLASS I MOLECULES

MHC class I molecules are glycoproteins consisting of a heavy chain, about 350 amino acids, and a light chain also known as β_2-microglobulin, about 100 amino acids (1). The human MHC is called HLA, that of the mouse H-2. Class I heavy chains as well as most other MHC genes are encoded on chromosome 6 in humans and on chromosome 17 of the mouse. The heavy chain consists of three extracellular domains, α_1, α_2 and α_3, a transmembrane region, and a cytoplasmic tail. β_2-microglobulin is noncovalently attached to the α_3 domain. The α_1 and α_2 domains form a peptide binding groove made up of a β-pleated sheet and two alpha helices, as has been revealed by X-ray analysis of HLA-A2, Aw68, and B27 crystals (2–5). The extension of the peptide-accommodating groove is about 1 × 2.5 nm, and it holds peptides in roughly extended conformation of about 8 to 10 amino acids (5–7). The peptides are tightly bound in the groove; both N and C termini are buried and are interacting with MHC residues. In addition, certain side chains of peptide amino acid residues interact with corresponding MHC residues forming pockets, whose location and shape varies with the allelic forms of the molecule. The extreme polymorphism of MHC class I genes—both mice and humans

have a couple of dozens if not hundreds of alleles at each of the three class I loci, and one allele may differ from the other by 40 amino acids (1)—is reflected in different peptide-accommodating specificities of the respective grooves (2–7).

APPROACHES TO ANALYSIS OF CLASS I-ASSOCIATED PEPTIDES

The classical experiments showing that virus-specific T cells recognize viral antigen in the context of MHC class I molecules were those of Zinkernagel & Doherty (8). Such T cells could also be stimulated with synthetic peptide (9), and Townsend and colleagues localized these antigens on fragment of intracellular viral proteins, for which synthetic peptides bound to uninfected cells can also be substituted (10, 11). Isolation of naturally processed peptides recognized by class I-restricted T cells was not reported until 1990 (12). Two principle methods are used to isolate such peptides.

Acid Extraction of Peptides from Whole Cells

If cells are mechanically destroyed and treated with trichloroacetic acid the majority of material precipitates, whereas small peptides including class I ligands stay in the supernatant. The heterogeneous mixture of peptides and other molecules is then separated by gel filtration and HPLC, and the individual fractions are tested for recognition by T cells (12). This is done by incubating ^{14}C labeled target cells expressing the relevant MHC molecule, but not the relevant antigen, with the individual HPLC fractions. This process is followed by incubation with cytotoxic T cells specific for an antigen expressed by the cells used to prepare the extract. Such an approach is useful for isolation and characterization of peptides for which specific T cells are on hand. The first naturally processed T cell epitopes have been isolated this way; some of these were identified by biochemical comparison with synthetic peptides (12–14). An advantage of this approach is that it detects not only peptides that had been bound to MHC molecules in the intact cells, but also all other peptides that have the capacity to bind to MHC molecules and are recognized by T cells (15, 16).

Acid Extraction of Peptides from Purified MHC Molecules

Isolation of MHC class II molecules by immunoprecipitation followed by acid extraction of associated peptides has been reported by Demotz and colleagues (17). The first report on peptides isolated from purified class I molecules was by Van Bleek & Nathenson (18). The approach is to lyse cells with detergent, to precipitate MHC molecules by antibodies bound to a solid phase, and to dissociate peptides from MHC molecules by acid

treatment. Dissociated peptides are then separated by HPLC and can be analyzed by T cells, as with the previous approach. This led to identification of a natural vesicular stomatitis virus (VSV) epitope (18). Such MHC-eluted peptides are more readily accessible for sequencing due to less contamination with material not associated with MHC molecules. Partial sequence information of a class I-associated viral epitope has been derived by biosynthetic labeling of VSV-infected cells with radioactive amino acids Tyr and Leu; thus, the position of these residues but not of other residues was determined by this sequencing approach, confirming the identity of the VSV epitope (18). Direct sequencing of peptides, by Edman degradation and/or by mass spectrometry, allowed determination of complete ligand sequences of unknown protein origin (19-21).

THE ANTIGENIC SYSTEMS USED TO CHARACTERIZE NATURAL PEPTIDES RECOGNIZED BY CLASS I-RESTRICTED T CELLS

Peptides Derived from Foreign Proteins Expressed from Transfected Genes

Mouse cells expressing foreign proteins from transfected genes, like HLA, β-galactosidase, or ovalbumin, readily induce class I-restricted CTL in syngeneic mice and are recognized by such T cells (e.g. 22-25). The peptides recognized can be narrowed down using synthetic peptides or fragmented protein, similarly as for virus-specific CTL. The natural peptide from β-galactosidase, as recognized by L^d-restricted CTL, has been isolated from β-galactosidase-transfected tumor cells (12).

Similarly, the natural peptide recognized by K^b-restricted CTL specific for ovalbumin was isolated, this time using purified K^b-molecules from transfected cells as source of peptide (26).

Minor Histocompatibility Antigens

Grafts exchanged between MHC-matched individuals can still be rejected, if other genes are different. Such non-MHC gene products leading to graft rejection are called minor histocompatibility antigens (27). Numerous genes of the sort are scattered throughout the mouse genome, many of which have been mapped and numbered: H-1, H-3, H-4, and so on. These antigens are recognized by MHC-restricted T cells (28). Minor H genes are also known in humans (29). The general idea is that such antigens are peptides derived from normal but polymorphic cellular proteins presented by MHC molecules, as first proposed by Townsend (10, 30, 31). Only few minor H genes have been identified so far; almost all are rather exceptional.

One is β_2-microglobulin whose allelic forms can be recognized by CTL (32-34), probably by conformational determinants, and not by recognition of β_2-microglobulin-derived peptide sitting in the MHC groove. Others are different allelic forms of a mitochondrially encoded gene (ND1, a subunit of NADH-dehydrogenase), peptides of which are presented by an odd class I molecule, Hmt, that is encoded telomeric of the H-2 region (35). (In addition, retroviral gene products can behave as minor H antigens—35a). Of those classical minor H gene products encoded autosomally and presented as peptide by classical MHC molecules, only one has been identified so far—the myxovirus resistance protein Mx recognized by K^b-restricted T cells (36). However, an additional one has been isolated as a peptide (37), and several others as natural MHC class I ligands, like the K^b-restricted H-4^b antigen, the Y-chromosome encoded H-Y antigen (D^b-restricted) as well as an HLA-B35-restricted human minor H antigen (12, 15, 38).

Peptides Recognized by Alloreactive CTL

The trait leading to discovery of MHC genes was the strong rejection of MHC incompatible grafts in mice (caused by T cells) (1, 39). This reaction has its in vitro correlate in the strong activation of T cells confronted with stimulator cells expressing MHC. The specificity of such "alloreactive" T cells is rather heterogeneous. Some of such T cells might be peptide-independent, whereas a portion is clearly peptide-specific or at least peptide-dependent (16, 40-51). The analysis of naturally processed peptides recognized by several alloreactive CTL lines nominally directed against the H-2K^b-molecule has provided some insight into general aspects of protein processing in the class I pathway (16), a point discussed later. In addition, these experiments explained the high frequency of alloreactive T cells against any given foreign MHC molecule by the high number of combinatorial foreign MHC/foreign peptide complexes on any given foreign cell (16, 52, 53). The term "foreign peptide" in this context deserves closer consideration, a point discussed in the paragraph on self-tolerance.

Viral Peptides

The first naturally processed T cell epitopes to be identified were of viral origin. Identification was possible because the antigen recognized by the respective CTL had been narrowed down by transfection of individual viral genes, or truncated genes, and by analysis of CTL using synthetic peptides according to viral sequences, as pioneered by Townsend and colleagues (10, 11). The D^b-restricted influenza nucleoprotein nonapeptide ASNENMETM (Nucleoprotein 366-374) as well as the K^d-restricted non-apeptide from the same protein, TYQRTRALV (NP 147-156), were identi-

fied as natural T cell epitopes (13, 14) by comparison with longer synthetic peptide preparations known to contain the respective epitopes (11, 54). At the same time, van Bleek and Nathenson identified a K^b-restricted octapeptide of VSV nucleocapsid protein, RQYVYQGL, by eluting the natural peptide from purified K^b molecules (18). Only one additional natural viral epitope, the L^d-restricted nonapeptide YPHFMPTNL of mouse cytomegalovirus (MCMV) (55), has been identified to date (June 1992), although for many other cases likely candidates are known.

An important subsidiary point of the above mentioned work on influenza peptides was that the biological effects of a given synthetic peptide prep-aration may be due mainly to byproducts occurring in minute amounts. Crude synthetic IASNENMETMESSTLE (influenza nucleoprotein 365-380), for example, contained a very small amount of ASNENMETM that did not give an OD 220 signal in the HPLC profile and still was the most active fraction recognized by D^b-restricted, influenza-specific CTL (13, 14). This is due to the extremely high biological activity of natural epitopes, detectable in CTL assays down to the femtomolar range. This point was later confirmed with different peptide preparations and assays (56).

Peptides Recognized by Tumor-Specific CTL

Certain tumors in the mouse as well as in humans can be efficiently recognized by tumor-specific or, at least, tumor-directed CTL. Several genes coding for antigens recognized in such situations have been identified by the pioneering work of T. Boon and colleagues, for example, the antigens recognized on P815 mastocytoma cells of DBA/2, or of variants of these cells (57-60). Similarly, the antigen recognized by human melanoma-specific T cells has been identified (61). Although in these cases the genes as well as the approximate location of the T cell epitopes is known, the natural MHC ligand recognized by tumor-specific CTL was identified in only one case so far: The K^d restricted nonapeptide KYQAVTTTL is recognized by CTL specific for an immunogenic variant of P815 tumor cells (62).

THE RULES FOR PEPTIDES PRESENTED BY CLASS I MOLECULES

Pool Sequencing of Peptide Gemische Eluted from Class I Molecules

The natural K^d-ligand TYQRTRALV (derived from influenza nucleo-protein), mentioned above, contains a Tyr (Y) residue, similar to several synthetic peptides known to contain K^d-restricted T cell epitopes and known to bind to K^d. Tyr is important for K^d-binding, as well as a Leu or

Ala residue 8 positions apart, as shown by J. Maryanski and coworkers (63). Alignment of the natural nonamer with those synthetic peptide sequences according to their Tyr residues suggested that all natural K^d-ligands might be nonamers with Tyr at position 2, and an aliphatic residue like Ile, Leu, or Val, at the C-terminus (14). This hypothesis was essentially confirmed when the natural K^d-ligands isolated from P815 tumor cells were sequenced as a pool (19; Table 1). Similar approaches were also useful for other class I molecules, like K^b, D^b, and HLA-A2 (19). The results indicated that each MHC class I allelic product has its own peptide speci-ficity, a peptide motif, characterized by an allele-specific length of 9 (K^d, A2), or 8 (K^b) amino acid residues. Certain positions, like position 2 of K^d-ligands, are occupied by residues with similar side chains; such positions are called anchors. Positions frequently but not always occupied by similar residues are called auxiliary anchors. The characteristics of the ligands of a given MHC allelic product are summarized as peptide motifs (19). The peptide motifs known so far (mostly determined by pool sequenc-ing) are those of H-2K^d, K^b, D^b, K^k, K^bm1, HLA-A*0201, A*0205, and B*2705 and are compiled in Tables 1 through 6.

Comparing these peptide motifs with synthetic peptides containing T cell epitopes suggested that most synthetic peptide epitopes determined so far were longer than the respective natural ligand. In addition, such comparison suggested in some cases that the allele-specific peptide length is not so much determined by the number of amino acid residues, e.g. 9 for D^b-ligands, but rather by the spatial length of peptides as mounted in the MHC groove, since, especially for some Pro- and Gly-containing T cell epitopes, the aliphatic C-terminus could not be aligned at position 9 but rather at position 10 or 11 (Example: SGPSNTPPEI of Adenovirus) (19). It was suggested that certain residues like Pro (inducing kinks in peptide stretches) or combinations of Pro and Gly might contract the spatial lengths of peptides (19). Although natural class I ligands with more amino acids than the nominal allele-specific residue number have not been determined yet, optimal binding as well as T cell recognition of a Pro-containing D^b-restricted epitope with 10 instead of 9 residues (64) and of a K^b-restricted peptide with 9 instead of 8 residues (35a, 56, 65) suggested that the above assumption was correct. The notion was indeed proven recently by analysis of monopeptidic class I crystals (65a,b).

Sequencing of Individual Peptides Eluted from Purified MHC Molecules

Information on the ligand specificity of individual MHC molecules can also be derived by comparing a set of individual peptides eluted from the

Table 1   K^d-motif (Ref. 19)

	Position								
	1	2	3	4	5	6	7	8	9
Anchor residues*		**Y**							**L**
									I
Frequent residues	N		P	M	K	T			
	I			F	N				
	L								
Also observed	K	F	A	A	V	H	P	H	V
	A	H	S	I	I	K	E		
	R		V	E	D	M	D	K	
	G	R	D	I	Y	E	V		
	V	S	R	L	V	Q	F		
	T	F	H	S	R	S	R		
	G	E	R	T	L	A	L		
	Q		G	T	T				
	K		E	H	Y				
	I								

Natural ligands	Source	Ref.
T Y Q R T R A L V	Influenza nucleoprotein	13,64
S Y F P E I T H I	Protein tyrosin kinase JAK1	19,66
K Y Q A V T T T L	Tumor antigen of P815	102
G Y K D G N E Y I	Listeriolysin (L. monocytogenes)	70

Likely ligands		
R V L E N G K E T L	HLA-A2-OH	19,123
R Y L K N G K E T L	HLA-Cw3	19,82
S V I P S A E K I	Plasmodium circumsporozoite protein	19,66,124
S Y Y P S A E Q I	Plasmodium circumsporozoite protein	19,66,123

* Anchor positions in bold.
* Sequences that (i) fit to the motif and (ii) are contained in synthetic peptides recognized by the respective T cells, or correspond to the synthetic peptide with highest biological activity.

Table 2   D^d motif (Ref. 19)

	Position								
	1	2	3	4	5	6	7	8	9
Anchor residues					R				I
Frequent residues	M	I	K			L			L
		L	E			F			
		P	G						
		V	V						
Also observed	A	A	G	G		A	D	F	L
	N	G	R	T		V	E	H	
	I	D				T	Q	K	
	F	S				V	V	S	
	P					E	T	V	
	S					E	V	T	
	I					S		G	
	V					V	K		
						Q	L		
						R			
						S			

Natural ligand	Source	Ref.
A S N E N M E T M	Influenza nucleoprotein	7,13

Likely ligands		
S G P S N T P P E I	Adenovirus	19,94
A G V E N P G G Y C L	LCMV	19,126
S A I N N Y A Q K L	SV40	127
C K G V N E E Y L	SV40	127
Q G I N N L D N L	LCMV	128
F Q P Q N G Q F I	FLINP 1968	78
A B N E N M D A M		

grooves. This approach has the advantage that the protein of origin can be tracked down. The first natural MHC ligand to be directly sequenced was SYFPEITHI (19), a prominent self-peptide occupying about 5% of K^d molecules on P815 tumor cells. A matching peptide stretch, SFFPEITHI, was found in a human sequence, protein tyrosine kinase JAK 1 (66). Thus, the natural K^d ligand suggested that the corresponding mouse JAK 1 sequence was SYFPEITHI, a notion confirmed in the meantime by sequencing of the mouse gene (A. G. Harpur, A. Ziemiecki.

Table 3 K^b-motif (Ref. 19)

	Position[a]							
	1	2	3	4	5	6	7	8
Anchor residues					F			L
					Y			
Frequent residues					Y			M
Also observed	R	N	F	R	T	S	E	
	L	Q	I	D	I	Q	V	
	L	I	L	E	E	K		
	S		K		S	Q		
	A		T	Q				
	H		V	P				
	N							

									Source	Ref.
Natural ligands	R G Y V Y Q G L								VSV	10
	S I I N F E K L								Chicken ovalbumin	23
	H I Y E F P Q L								Rat protein of P815	134
Likely ligands	F A P G N Y P A L								Sendai virus	58
	S I L E F A R L								HSV glycoprotein B	K. Rammensee, pers. communication

[a] Auxiliary anchor residues underlined.

A. F. Wilks, K. Falk, O. Rötzschke, H.-G. Rammensee, unpublished observation).

The approach of directly sequencing individual MHC ligands has been followed up by several laboratories for both class II (67–69) and class I (20, 21, 70). The peptide motif of HLA-B*2705 has been determined this way by analyzing 11 complete or partial sequences of normal B27 ligands (20). The technique was greatly improved by using mass spectrometry for microsequencing, yielding complete or partial sequences of some 9 HLA-A2 ligands (21), that entirely confirmed the A2 motif as determined by genmisch sequencing. The natural ligands of the different class I allelic

Table 4a K^d-motif[a]

	Position								
	1	2	3	4	5	6	7	8[b]	
Anchor residues		E						I	
Frequent residues			K						
			N						
			Y						
			M						
Also observed	V			G	L	A	N	T	
	F			L		G	K		
				J	L	P	H		
				F	P	Y			
				H	R				
				Y	S				

										Source	Ref.
Likely ligands	F E S T G N L I									Influenza Haemagglutinin 2	123
	F E A N G N L I									Influenza Haemagglutinin 1	123
	I E G G W T G M I									Influenza Haemagglutinin 2	123
	B E F L L E K R I									SV40 T antigen	123
	Y E M D I E K K I									Plasmodium chorungosporozoite protein	123
	S D Y E G R L I									Influenza Nucleoprotein	123
	D E L D Y E N D I									Plasmodium circumsporozoite protein	129

[a] M. Aboth, K. Falk, O. Rötzschke, S. Stevanovic, O. Aung, and H.-G. Rammensee, submitted.
[b] The C-terminal residue appears to be invariantly Ile (the C-terminus can be at least as well as at 9 although the majority of natural ligands are probably C-terminal.

products—detected by direct sequencing or by comparison with synthetic peptides—are also compiled in tables 1 through 6 as well as some likely natural ligands. In addition, two naturally processed ligands have been described for L^d-molecules, YPHFMPTNL and LSPFPFDL (31, 55). Together with partial information on the L^d-specificity from a pool-sequencing attempt (19), and with information on an optimal synthetic T cell epitopes (59, 71) the L^d motif is likely to be XPXXXXXXL, with other aliphatic residues allowed at 9, and Ser tolerated at 2.

Table 4b. K^d-motif[a]

	Position							
	1	2	3	4	5	6	7	8
Anchor residues								
Frequent residues		E						K
Also observed		Q					H P A R	
		D					Q R Y	
		P					S K	
		M						
		F						
		Y						

[a] M. Neum, K. Falk, O. Rötzschke, S. Stevanovic, G. Jung, and H.-G. Rammensee, submitted.

A method slightly at variance to direct sequencing of MHC ligands is to label cells metabolically with individual radioactive amino acids before peptide elution and to determine the relative position of that residue within the ligand. This approach has been used for identification of a natural VSV epitope (18), to confirm frequent usage of Tyr at positions 3 and 5 and Leu at 8 of the K^b-motif and to determine the position of these residues in several natural ligands of K^bel and K^bm molecules (72).

Prediction of Natural T Cell Epitopes Using the Motifs

If the sequence of a cellular protein of immunological interest is known, comparing the sequence with the allele-specific peptide motif of a relevant MHC molecule indicates which stretches of the protein are likely to be natural MHC ligands. An example is chicken ovalbumin expressed in H-2^b mouse cells. The ovalbumin epitope recognized by K^b-restricted T cells had already been narrowed down to one contained in the sequence IINFEKL TEWTSSNVNEER (chicken ovalbumin 258–276) (24, 73). The K^b-restricted peptide motif requires an octamer with an aromatic residue at position 5 and an aliphatic one at 8 (19). Since the only aromatic residue in the above sequence is the Phe (F) at position 4, an additional residue at the N-terminus, Ser, was required by the motif, to result in the octamer SIINFEKL. This prediction was experimentally confirmed by elution of the natural ovalbumin peptide from K^b molecules, followed by biochemical comparison to synthetic SIINFEKL (26). Similarly, the K^b-restricted pep-

Table 5a. HLA-A2.1-motif (A*0201) (Refs. 19, 20)

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor residues		L							V		
Frequent residues		M				E		V	K		
Also observed		A G I I A E L									
		V P K L V S									
		F O V T H B									
		K P T R O V Q									
	M	U S Q A A									
	V	S V F P H									
	A	R V									
	S	Q V									
	G	V A									
	T	L A									
	N	R									
	W	D									
Natural ligands		S X P B G G X G V								Human IP 30 signal peptide	64
		L L D V P T A A V								Human IP 30 signal peptide	63
		L L D V P T A A V Q A								Human IP 30 signal peptide	95
		L L D V P T A A V								P 51, melanocyte-specific protein M	96
		G X V P F X V S V									89
		S L L P A I V E L									89
		S X X V R A X E V									89
		K X N E P V X X K									89
		Y L L P A I V H I									99
		T L W V D P Y E V									99
Likely ligands		I L K E P V H G V								HIV reverse transcriptase	19
		Q I L G F V F T L								Influenza matrix protein	59,139,135
		I L G F V F T L T V								Influenza matrix protein	39
		K L Q E F Y N Q M								Influenza BNP	131

X: Leu or Ile.

Table 5b HLA-A*0201-motif (Ref. 136)

	Position								
	1	2	3	4	5	6	7	8	9
Anchor residues									L
Frequent residues		V	Y	G	V	I			K
		L	P	E	Y				
		I	F	D	L				
		Q	I	I					
Also observed		M	K	Y	Q				
		N	T						
		L							
		A							
		R							

tide recognized by CTL specific for a variant of P815 tumor cells was predicted and demonstrated to be KYQAVTTTL (62). In the case of a Listeria protein, Listeriolysin, the epitope recognized by K^d-restricted CTL had not been narrowed down before by synthetic peptides or truncated genes. The K^d-restricted peptide motif indicated 8 nonamers in the Listeriolysin sequence as candidates for K^d-ligands, one of which, GYKDGNEYI, was indeed the correct one (73a). Thus, the MHC allele specific peptide motifs are useful for identifying natural T cell epitopes in proteins of immunological interest.

Allele-specific Peptide Motifs Are Not Just "Binding Motifs"

The majority of peptides that have been described so far to bind to class I molecules do not adhere to the respective peptide motif (e.g. 14, 63–65, 74–78a). This reflects the capacity of class I molecules to bind peptides of between 4 and 28 amino acids, no matter whether they do or do not contain the relevant motif. However, the binding of the natural ligand is in most cases several orders of magnitude better than binding of the "wrong" peptides (13, 14, 56, 79). Because the peptide motifs were determined not by screening for peptides that are the best binders but rather by analyzing the MHC ligands occurring naturally, these motifs incorporate each single step required for a peptide to become a natural MHC

Table 6 HLA-B27-motif (Ref. 20)

	Position									Source	Ref
	1	2	3	4	5	6	7	8	9		
Anchor residues		R									
Also observed	R		Y	Q	K	S	T	E	K		
	Q		I	K	E	I	V	K	R		
	K		V	L	P	V	G	R	Y		
	A		W	D	G	A	I	L	F		
			S	N	R	L	E	R	L		
			I	E	P	R	Q				
			F	T	A						
			L	P	A						
Natural ligands	R	R	Y	Q	K	S	T	E	L	Human Histone H3, H3.3	20
	R	R	I	K	E	I	V	K	K	Human Hsp 89a	20
	R	R	Y	K	E	V	V	K	A	Human Hsp 89b	20
	R	R	W	L	P	A	G	d	a		21
	R	R	S	K	E	I	T	V	R	Human elongation factor 2	20
	G	R	I	D	K	P	I	L	K	Human RNA-dependent RNA-helicase	20
	f	R	Y	N	G	L	I	H	r	ribosomal protein	20
	K	R	F	E	Q	L	T	Q	R	Rat 60 S ribosomal protein L28	20
	R	R	I	S	G	V	D	R	Y		20
	A	R	L	F	G	I	R	A	K		20
Likely ligands	S	R	Y	W	A	I	R	A	K	Influenza nucleoprotein	152
	S	R	Y	W	A	I	R	T	R	Influenza nucleoprotein	20, 152
	K	R	W	I	I	L	G	L	N K	HIV gag p24	20, 155
	G	R	A	F	V	T	I	G	K	HIV gp120	20

a Compiled from the single peptides shown below.
b Lower case letters: residues of lower confidence.

ligand. One of these steps, of course, is binding; others, however, include the processing of peptides that must be influenced by the specificities of the enzymes involved, and probably also the transport of either proteins

or peptides between cellular compartment (see below). It is, therefore, scientifically not exact, and sometimes rather misleading, to talk of allele specific peptide motifs as "binding motif". In the case of K^d, on the other hand, a true peptide binding motif has been established by Maryanski and coworkers, and it has the same basic characteristics as the motif based on natural ligands (80). Thus, peptide binding motifs and natural ligand motifs of MHC molecules may coincide but should not be expected to do so a priori.

Peptide Specificity of "Nonclassical" Class I Molecules

In addition to the "classical" class I genes, HLA-A, B, C, and H-2K, D, L, many other class I genes are encoded on the respective chromosome; some of them are expressed on the cell surface (1). Some of those "nonclassical" class I molecules have been shown to present antigen in a few cases (e.g. 34, 81, 82). For the Hmt molecule, which presents a mitochondrially encoded minor H antigen, Mta, to CD8+ T cells (35), studies with synthetic peptides have indicated that this molecule is able to bind peptides formylated at the N-terminus (83). Although natural Hmt-ligands have not been identified yet, these data indicate that Hmt molecules are specialized in presenting formylated peptides that might occur as fragments of proteins from prokaryotes or mitochondria (83). Analysis of natural ligands of another nonconventional class I molecule, glycophospholipid-anchored Qa-2, indicated a peptide motif similar to that of ordinary class I molecules, except that it seemed to be much more stringent in its specificity. According to this study, Qa-2 ligands are nonapeptides with two anchors (His at 7 and L, I, or F at 9) and four auxiliary anchors (O. Rötschke, K. Falk, S. Stevanović, B. Grahovac, M. Soloski, G. Jung, and H.-G. Rammensee, submitted); thus, only a relatively small number of nonapeptides would fit to Qa-2. It appears, therefore, that at least some of the nonclassical class I molecules, like Hmt and Qa-2, are peptide receptors of narrower specificity than ordinary class I molecules.

HOW DO CELLS MAKE THE PEPTIDES?

This question can be answered by a plain "not known" (June 1992). However, there is a long list of observations that give hints towards the mechanisms involved. Consideration of some of these observations, and disregard of others, has led to a view now widely accepted although unproven.

The Common Dogma

A widely published view of the mechanism for peptide loading of class I molecules is the following (see 84–101). Proteins are cut into the correct

8-mers or 9-mers by proteasomes in the cytosol. The resulting peptides are then transported to the ER by ATP-dependent peptide transporters encoded by TAP 1 and TAP 2 genes located in the neighborhood of MHC class II genes. In the ER, class I molecules select the peptides fitting to the respective motifs and bring them to the cell surface. There is no doubt on the last step, i.e. that class I molecules transport peptides to the plasma membrane. There is also no doubt that TAP 1 and TAP 2 genes are essential for proper function of class I molecules (88, 94); indeed, the TAP gene products influence the peptide pattern that could be eluted from a rat class I molecule (100). The other parts of the dogma, including the compartmentalization, are merely speculative. There is no evidence so far that "peptide transporters" really do transport peptides, nor is there an indication on the identity of peptides potentially involved, although a good dozen of papers or editorials have been published in *Nature* or *Science* so far on those genes or molecules. Another explanation (although not very likely) is that the latter act as MHC-flippase, that is to toggle class I molecules from the ER lumen to the cytosol and back, as has been acknowledged in a recent review (99). The following paragraphs deal with observations that do not easily fit with the above model.

Protein But Not Final Size Peptide Is Found in the Cytosol

One of the first naturally processed T cell epitopes to be isolated (but not identified) was derived from β-galactosidase expressed from a transfected lacZ gene in P815 tumor cells (72). The transfectant, P13.1, contains high amounts of β-galactosidase in the cytosol, as detectable by β-galactosidase enzyme activity. However, the naturally processed peptide recognized by L^d-restricted T cells that can be found in whole cell extracts as well as in the membrane fractions, was not detected in the cytosol (12 and O. Rötschke, K. Falk, H.-G. Rammensee, unpublished). Thus, if the final sized peptides are really produced in the cytosol, their duration or half-life in this compartment must be too short to allow detection.

Occurrence of Peptides in Whole Cell Extracts Is MHC Dependent

As mentioned above, whole cell extracts from β-galactosidase expressing P13.1 cells (H–2^d) do express the L^d-restricted β-galactosidase T cell epitope. EL4 tumor cells (H–2^b) transfected with the same gene and expressing even more β-galactosidase activity in the cytosol do not contain the L^d-restricted peptide (12 and K. Falk, O. Rötschke, H.-G. Rammensee, unpublished). This result indicated that coexistence of the correct class I molecule is required for occurrence of a given T cell epitope, since EL4 cells do not express L^d. This notion was confirmed with several

other antigens, including minor H and viral antigens (15, 16, 38, 55). MHC dependency of the cellular peptide content of cells was formally proven by comparing the peptides extracted from class I wild type and mutant mice (15) and was confirmed by analyzing cells transfected with class I genes (38, 102) as well as with tissue from transgenic mice (103). A semiquantitative study, using the K^b-restricted minor H peptide, H-4^b, indicated that a high copy number (5000-fold over detection limit) of K^b-restricted H-4^b peptide is present in an extract from 10^6 P815 cells transfected with K^b, but no detectable peptide is detected in untransfected P815 cells (102). These data suggest that, at a given point, not a single detectable copy of the final sized H-4^b peptide is present in cells not expressing K^b. An exception to the strict MHC dependency of peptide occurrence in whole cell extracts has been reported recently for one L^d ligand recognized by alloreactive T cells (51).

Precursor/End Product Relationship?

The minor H antigen, H-4^b, mentioned above, shows a peculiarity informative for general considerations on antigen processing. In peptide mixtures eluted from purified K^b molecules of H-4^b expressing cells, T cells recognize but one H-4^b peptide (called H-4^b main peptide) (15). In contrast, however, H-4^b specific CTL recognize two peptides in whole cell extracts of K^b expressing H-4^b cells (15, 102). These two peptides can be distinguished by their behavior on HPLC; one of them is identical to the H-4^b main peptide, the other is called the "H-4^b prepeak." If whole cell extracts of cells lacking K^b are analyzed, only one H-4^b peptide is found, the H-4^b prepeak. Thus, the H-4^b main peptide is strictly MHC dependent, like most other class I restricted peptides (see previous paragraph), whereas the H-4^b prepeak peptide (being not a natural K^b ligand) is MHC independent in its occurrence in whole cell extracts. We speculated that the two peptides may be linked by a precursor/end product relationship (15). The MHC independent one could be a precursor peptide cut out from the H-4^b protein by enzymes that are independent of MHC molecules. This larger precursor—which happens to be able not only to bind to K^b but also to be recognized by K^b-restricted CTL—is then transported to the compartment of class I loading, binds to K^b, and then is trimmed to give the final sized ligand, according to our hypothesis (7, 15, 19). Alternatively, the MHC independent peptide might be derived from another proteolytic pathway unlinked to class I-restricted processing.

A similar situation—two peptides found in whole cell extracts, only one of which is MHC dependent and a natural class I ligand—was found for the peptide-specific, alloreactive, K^b-specific CTL clone 27.5 (16). In addition to an MHC-dependent peptide, this clone recognizes a peptide

present in spleen cells of all mouse strains, in human cells (Jurkat), and even in yeast or earthworm cells, indicating that this peptide—obviously derived from a conserved protein—is really MHC-independent.

Considering these data, one could speculate that the final size peptides—the class I ligands—are always derived from MHC-independent larger precursor peptides, and that detection of the latter depends on the chance that a given T cell happens not only to recognize the natural class I ligand, but also its larger precursor.

An alternative explanation for the observed MHC-dependency of peptide occurrence in cells, however, would still be "determinant protection" of entirely preprocessed peptides by binding to MHC molecules (15, 104, 105). Such a mechanism, however, would pose a principal problem that is discussed in one of the following paragraphs.

The Location of a Peptide Stretch Within a Protein Does Not Influence the Identity of Processed Peptides

The sequence coding for a natural L^d ligand, YPHFMPTNL, from murine cytomegalovirus immediate early protein, was inserted into several sites of an unrelated gene, with or without linker sequences, and the construct was expressed in cells (55). In all cases, the natural L^d ligand was the same nonapeptide, although considerable quantitative differences were observed. In another study, when viral epitopes were inserted into various protein sites, the natural ligands were not identified, but recognition of all the different constructs by the same T cells suggested that the same epitope was processed in all cases (106). Thus, although the data base for these kind of experiments is still small, it appears that the identity of a processed class I ligand is generally independent of the flanking amino acid residues in the protein of origin, and that all the informations for cleavage sites must be within the ligand sequence. In still another study, certain flanking sequences of epitope-containing minigenes transfected into cells did not allow T cell recognition (107); because the identity of the corresponding ligands had not been identified, the data are still consistent with influence of flanking sequences on quantity but not identity of peptides.

Human Cells Know How to Make the Peptides for Mouse Class I Molecules

The alloreactive, K^b-specific CTL line 26T0-3 not only recognizes H-2^b mouse cells, but also human Jurkat tumor cells transfected with K^b (16). A peptide recognized by this CTL line can be isolated from both mouse and human cells expressing K^b. The peptide is probably derived from a protein conserved between mouse and human. Biochemical comparison of the peptide from mouse cells with that from human cells indicates that

both are identical (108). Another ligand, recognized by CTL clone 9.6, yielded similar data, thus confirming the notion.

Ovalbumin-specific, K^b-restricted CTL recognize the ovalbumine peptide SIINFEKL on K^b molecules of mouse cells (26), a point mentioned previously. Human HeLa cells transfected with both the ovalbumin gene and the K^b gene are also recognized by above CTL, and SIINFEKL is a natural ligand of K^b molecules of those HeLa transfectants (O. Rötzschke, K. Falk, N. Shastri, H.-G. Rammensee, submitted).

Thus, at least in those three examples, human cells process the same peptides from a given protein, if the relevant class I molecule, K^b in the examples given, is expressed in the cells. This indicates that the mechanism cutting proteins into peptides for class I loading—in its specificity—is also the same between different mouse strains, and also between different species. Moreover, this specificity is also the same between different tissue, and also between different mouse strains, since minor H peptides like B-4^b or H-Y appear to be the same no matter which tissue or mouse strain with correct MHC expression is analyzed (15, 103). Even tissue like brain tissue, normally almost class I negative (and thus, minor H peptide negative), can be induced to express the same H-4^b peptide as other tissue upon expression of transgenic K^b (103).

Extrapolating these data suggests that a single mouse brain cell, for example, is instantly able to provide any peptide ligand required not only by any of the hundreds of mouse class I molecules but also by the thousands of different class I molecules in humans and all other mammals. The commonly accepted model for antigen processing—postulating complete peptide processing before contact with class I molecules—would require a constant pool of ten of thousands of processed ligands fitting to all the class I molecules of all mammals, and this even in cells like brain cells that don't even express their own class I genes.

In addition to this conserved, nonpolymorphic general mechanism, however, polymorphism in peptide loading that has been shown for the class I system in rats (100) and suggested for B27 molecules in humans (109), might exist as an epiphenomenon. One possibility is also that the conserved mechanism works for all class I molecules requiring a hydrophobic C-terminus of peptide ligands, whereas the polymorphic mechanism works for class I ligands with a charged C-terminus, as for B27 ligands. (The B27 molecule appears to have a flexible pocket for the C-terminal peptide side chain able to accept either hydrophobic or charged residues; 110). Thus, the observed functional polymorphism might be due to the presence or absence of transacting factors (TAPs or proteasomes?) that are able to generate, transfer, or treat otherwise peptides terminated by charged residues.

A Model for Peptide Processing

Some of the above observations do not easily fit into the commonly accepted model on peptide processing. We have, therefore, proposed an alternative model that would explain those observations (7, 15, 19). The main point is that it assumes an instructive role for class I molecules in processing. We hypothesize that a protein is first degraded into (precursors) peptides larger than the final class I ligand (Figure 1). This can be, for example, in the cytosol, but is also conceivable in other compartments. The endopeptidase(s) doing the cutting could have a specificity such that cleavage occurs C-terminal of hydrophobic (Met, Ile, Leu, Val, Phe) or charged residues (a specificity covered by proteasomes); this way the precursor peptides would already have the correct C-terminus fitting to most class I motifs known so far. The resulting peptide precursors—if not produced in the ER itself, like leader peptides would be, as we had suggested (16)—would then be translocated to the site of class I loading, that is, the ER or the early Golgi. This might be done by the putative ATP-dependent peptide transporters which might be specific for the respective C-terminus or size, although there is no evidence so far that these molecules

Figure 1   Model for antigen processing in the class I pathway. For explanation, see text.

transport peptides and, at least in in vitro systems, it appears that peptides can cross the membrane of microsomes without requiring ATP (111–113). The precursor peptide would then bind with low affinity to fitting class I molecules, for example, first by contacting a conserved positive charge (Lys 146) of the groove with the peptide's C-terminus, and then by insertion of anchor residue side chains into the allele specific MHC pocket. The N-terminus, still too long, would then be trimmed to the allele-specific length by an unknown peptidase activity that could be an exopeptidase. As soon as the final size of the peptide is reached, it would snap into the groove and be bound with high affinity.

Proof for natural binding of larger peptides to A2 molecules as well as their trimming down to nonamers (from both ends, however) has been convincingly demonstrated by the work of Henderson et al, and Wei & Cresswell, who looked at natural peptides derived from a leader sequence (70, 98; Table 1). This work, in addition, confirmed our earlier hypothesis that leader peptides generated in the ER can serve as class I ligands (16).

It is clear that most elements of the alternative model described in this paragraph are highly speculative; since the model, however, can explain some of the intriguing facets of class I-restricted antigen processing, it may be useful as a working hypothesis.

HOW MANY PEPTIDES ARE PRESENTED?

Copy Number of a Given Peptide

Comparing the amount of natural VSV-derived peptide RYQVYQGL found on K^b molecules on infected cells with a defined amount of synthetic peptide, it was estimated that about 5% to 10% of K^b molecules of an infected cell are occupied with the VSV peptide (18). If we assume that 10^5 K^b molecules are expressed on a cell, this would amount to 10,000 RYQVYQGL peptide copies per cell. On the other hand, the K^d-restricted peptide TYQRTRALV of influenza is found only in 200 to 500 copies per infected cell (14). Similarly, the D^b-restricted influenza peptide ASNENMETM occurs in about 200 copies per cell (14), and the K^b-restricted ovalbumin derived peptide SIINFEKL as well as the tumor-associated K^d-ligand KYQAVTTTL is found in around 100 copies per respective cell (26, 62). The prominent self-peptide SYFPEITHI from K^d molecules of P815 tumor cells, representing the most abundant peptide species on K^d in this cell, is found in about 10,000 copies per cell (19). Thus, the copy number of individual peptides can be rather variable, judging from chromatography profiles of peptide pools eluted from class I molecules, it appears that on normal cells a few peptides are in the copy number range of SYFPEITHI, whereas most other peptides are at a much

lower rate, that is in the range of a couple of hundred copies. This fits well with mass spectroscopic analysis of A2-eluted ligands (21), and also fits well with the minimal number of synthetic peptides required to detect class I-restricted T cell recognition, if the peptides are added to the cells from outside (114, 114a).

The Number of Different Peptides per Cell

If a cell expresses 1 or 2×10^5 class I molecules of one kind, and the average copy number of a given peptide is 200, one would expect about 500 to 1000 different peptides per cell (14). Mass spectrometric analysis has allowed the detection of 200 different peptides eluted from A2 molecules, representing 50% of the A2-ligand population (21). Due to limits of detection, many low-copy number peptides could have been missed, and it was estimated based on the data that the number of peptides could exceed 1000. Thus it appears reasonable to assume that a given class I species presents around 1000 different peptides, with a rather broad range of copy numbers, however.

IMPLICATIONS FOR SELF-TOLERANCE

Self-Tolerance of Self-Peptides Is MHC restricted

The phrase "MHC restriction of self-tolerance" describes the events leading to the silencing of self-reactive T cell clones during their differentiation (115); they are dependent on the same kind of T cell recognition as in mature T cells, that is, recognition of MHC and antigen. Several years ago we learned that in bone marrow chimeras self minor H antigens do not induce tolerance if they are not presented with self MHC molecules, and normal mice do contain T cells reactive to self minor H antigens if presented on foreign MHC molecules (116–118). However, the antigens involved were not characterized at the molecular level, and the notion has been challenged (119). Peptide-specific alloreactive T cells allow us to readdress this question. The K^b-restricted CTL clone 27.B2, derived from a bm1 mouse (expressing K^bm1) recognizes a natural ligand of K^b that happens to be also a natural ligand of K^bm1. 27.B2 CTL recognize this peptide only if bound to K^b molecules, and not if bound to self K^bm1 molecules (16, 120). Similar settings have been found for other alloreactive CTL (50). It can be concluded that T cells can distinguish if the same peptide ligand is presented by different class I molecules, and also that T cells in the bm1 mouse have not been negatively selected against the complex of self-peptide and foreign MHC molecule. Thus, negative selection of T cells during differentiation must require recognition of peptide ligands bound to MHC molecules.

T Cells Are Only Tolerant to Self Peptides That Are Actually Presented

Negative selection only of T cells recognizing self-peptides presented by MHC molecules would implicate that T cells are not at all tolerant to self-peptide not naturally presented on self MHC molecules. This notion was confirmed experimentally. B6 T cells could readily be stimulated with synthetic peptides according to stretches of a self-protein, β-microglobulin, to result in peptide-specific, class I-restricted CTL (78). Those peptides did not contain K^b or D^b-restricted motif, and the CTL did not recognize H-2^b cells expressing β$_2$-microglobulin. Thus, the respective peptides are not presented naturally by cells but can bind to H-2^b molecules and induce CTL responses. Similar responses were obtained by stimulating T cells with trypsin-digested self-proteins (78). Thus, "self" for class I-restricted T cells is a rather limited selection of peptides derived from self-proteins, excluding the majority of self-protein sequences (14, 121). Consequently, peptides derived from that majority are treated as "foreign" by T cells, although derived from self sequences.

A COMPARISON WITH CLASS II

The approach of isolating and sequencing natural ligands of MHC molecules has also been applied for class II (67-69a). Differences distinguishing characteristics of class II ligands from those of class I are: (i) Class II ligands are longer, ranging roughly from about 12 to 20 residues. (ii) A given class II species can present peptides of various lengths, that is, strict allele specific length requirements are not observed. The class II peptide groove, therefore, appears to be open at both ends. (iii) Class II molecules appear to have allele-specific peptide motifs as well, although in the first publication on identified ligands, obvious motifs were not observed (67). More recent evidence indicates motifs containing two or three anchor-like positions that are not as stringent in occupancy requirements as is the case with class I molecules. A simplistic cartoon visualizing the principal differences between class I and class II ligands is in Figure 2.

CONCLUDING REMARKS

The function of MHC class I molecules is to display at the cell surface a selection of small peptides derived from cellular proteins. The selection is such that, on average, every cellular protein has a chance to participate with one or two peptides in this sampling (14, 121). Each member of a species displays her or his individual peptide selection, since the rules for

class I

Peptides from all endogenous proteins

class II

12-20

Peptides from proteins reaching the endosomal/lysosomal compartment

Figure 2 The principle differences between class I and class II ligands in a simplistic view.

peptide presentation are determined by the polymorphic MHC alleles (and in addition by the polymorphic *trans*-acting factors). An individual's T cell population is tolerant against his or her self-peptide collection, since all T cells recognizing one of these self-peptides are silenced during differentiation. If a new peptide appears, T cells can recognize it and attack the cell expressing it, eventually the death of the cell or necrosis of the tissue results. New peptides are likely to occur after a cell has been invaded by new genes, such as genes from viruses or other pathogens. Mutations in self-proteins could also lead to new peptides (122), although the chance that a point mutation hits a stretch carrying the motifs relevant for a cell is small. A third setting that may lead to occurrence of new class I ligands is the aberrant expression of proteins (60). In addition, the aberrant up-regulation of self-proteins could also lead to T cell recognition, since T cells can distinguish not only between different peptides but also between different quantities of the same peptide (55). Thus, peptides presented by class I molecules allow for control of the cell's interior through the immune system.

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THE IL–2 RECEPTOR COMPLEX: Its Structure, Function, and Target Genes

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Abstract

Proliferation of T lymphocytes is triggered by the interaction of IL-2 with its specific receptor following T lymphocyte activation. The receptor for IL-2 consists of at least three distinct subunits, the α chain (IL–2Rα), the β chain (IL–2Rβ), and the γ chain (IL–2Rγ). Although the role of IL–2Rγ in IL–2 signalling remains unclear, IL–2Rβ is the subunit critical for receptor-mediated signalling. Because IL–2Rβ lacks any apparent catalytic motifs, IL–2Rβ may be physically or functionally coupled to other signalling molecules.

Structure-function studies of IL–2Rβ have revealed that at least two distinct cytoplasmic regions of IL–2Rβ are involved in IL–2-induced cellular signalling. The "serine-rich" region of IL–2Rβ was identified as a region critical for IL–2-induced mitotic signalling from experiments in which IL–2Rβ mutant cDNAs lacking a particular cytoplasmic region or regions were expressed in an IL–3-dependent mouse pro-B cell line (BAF-B03). Meanwhile, another cytoplasmic region of IL–2Rβ, the "acidic" region, is responsible for its physical association with an src-family protein tyrosine kinase (PTK), p56⁴⁴ and is critical for activating the p56⁴⁴ PTK following IL–2 stimulation.

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The Epitopes of Influenza Nucleoprotein Recognized by Cytotoxic T Lymphocytes Can Be Defined with Short Synthetic Peptides

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Summary

A proportion of cytotoxic T lymphocytes (CTL) responding to infection by influenza recognize target cells that express the viral nucleoprotein. Recent work showed that CTL can recognize short overlapping regions of large nucleoprotein fragments expressed in transfected L cells. This led to the suggestion that CTL recognize segmental epitopes of denatured or degraded proteins in a similar way to helper T cells. One corollary of this idea is that CTL should recognize appropriate short peptides on the target cell surface. We demonstrate that the epitopes of nucleoprotein recognized by CTL in association with class I molecules of the major histocompatibility complex in both mouse and man can be defined with short synthetic peptides derived from the nucleoprotein sequence.

Introduction

Mice and humans retaliate with a vigorous cytotoxic T lymphocyte response to infection by influenza A viruses (reviewed by Askonas et al., 1982; Townsend and McMichael, 1985). Cytotoxic T lymphocytes (CTL) generally express the Lyt2 positive (CD8 in the human) surface phenotype and recognize and kill infected target cells that share class I molecules of the major histocompatibility complex (MHC) with the host in which the CTL developed (reviewed by Swain and Dutton, 1980; Dialynas et al., 1983; Blanden et al., 1975; Zinkernagel and Doherty, 1979). CTL may play an important role in limiting the spread of virus in vivo (reviewed by McMichael et al., 1983).

Little is known about the nature of the viral epitopes recognized by CTL on the surface of an infected target cell. Recently a major population of CTL from influenza infected mice have been shown to recognize murine L cells (a fibroblast line) that express the viral nucleoprotein (NP) in isolation after DNA mediated gene transfer (Townsend et al., 1984a). Recombinant vaccinia engineered to express influenza NP also has been used to demonstrate human (McMichael et al., 1986) and murine (Yewdell et al., 1985) CTL that recognize the NP molecule. Recent evi-

dence has shown that other nonglycosylated proteins of the virus can also be responsible for inducing cytotoxic T cells (Yewdell, personal communication).

These findings raised the question of how viral components that are not transmembrane proteins and do not have recognizable amino-terminal leader sequences are transported to the plasma membrane of the target cell, where CTL recognition is assumed to occur. To investigate this, L cells were transfected with a series of deletion mutants of the influenza NP gene in an attempt to identify sequences required either as epitopes recognized by CTL or as signals for membrane transport (Townsend et al., 1985).

Epitopes were localized by comparing L cells expressing large fragments of NP that overlapped over short regions. In this way two regions of sequence recognized by CTL were identified in a continuous segment of 59 amino acids between residues 328 and 386 of the molecule. No leader-like sequence could be located because nonoverlapping amino-terminal and carboxy-terminal fragments of NP were recognized equally well by appropriate CTL. This implied that the two ends of the molecule could be transported to the plasma membrane independently of each other. In addition, although all of the fragments of NP expressed in transfected cells could be recognized by specific CTL, some were no longer detected with antibodies that bound the complete folded molecule.

One possible explanation for these results is that nonmembrane viral proteins are degraded in the cytoplasm of the infected cell, producing short denatured peptides that are exported from the cell by some unknown mechanism. Such degraded viral proteins may then become available for recognition by CTL in association with class I MHC molecules in a way similar to that in which helper T cells recognize denatured or degraded proteins with class II molecules (reviewed by Grey and Chesnut, 1985; Unanue et al., 1984).

One of the main predictions of this proposal is that CTL should be able to recognize appropriate short synthetic peptides corresponding to linear regions of the NP sequence when they are added in vitro to the target cell surface. We describe experiments that define the epitopes of influenza nucleoprotein recognized by class I restricted CTL from both man and mouse using short synthetic peptides. Both the minimum length and the concentration required are similar to those required for recognition of protein antigens by class II restricted helper T cells. The results are consistent with the view that all somatic cells bearing class I molecules may be capable of degrading and presenting newly synthesized viral proteins to CTL.

Results

Murine and human CTL were tested for their ability to lyse ^{51}Cr-labeled target cells exposed to a variety of peptides derived from the nucleoprotein sequence (see Tables 1 and 3). Cloned murine CTL also were tested for their

Table 1. Amino Acid Sequences of the Nucleoproteins from A/PR/8/34 and A/NT/60/68 between Residues 329 and 386

	330	340	350	360	370	380
A/NT/60/68	LVWMACNSAAFEDLRVLSFIRGTKVSPRGKLSTRGVQIASNENMDAMESSTLELRSRYW					
A/PR/8/34		H		K V		ET

Sequences are from Winter and Fields, 1981, and Huddleston and Brownlee, 1982. The two peptide sequences recognized by class I restricted CTL are underlined.

proliferative responses to peptides in the presence of feeder cells and T cell growth factors. The ⁵¹Cr release experiments described below were performed either by incubating CTL and target cells in medium containing dissolved peptide (method 1), or by preincubating the target cells in a high concentration of peptide and then washing them extensively before exposure to CTL (method 2; see Experimental Procedures for details).

Recognition of Nucleoprotein Peptides by Murine CTL

Two clones of influenza A specific CTL were investigated for their ability to recognize peptides. Both were derived from C57BL mice (H-2b) and recognized their target antigens in association with the class I MHC molecule D^b.

Cytotoxic T Cell Clone F5

This cloned cell line has been described previously (Townsend et al., 1984a). F5 expressed the Lyt2 positive, L3T4 negative, Thy1 positive phenotype by indirect immunofluorescence with antibodies 53-6.7, GK 1.5, and 97-13 respectively (data not shown). It has been shown to be specific for the 1968 nucleoprotein, and the epitope recognized has been localized to a region of the molecule between amino acids 328 and 386 (Townsend et al., 1985).

Table 1 shows the relevant sequence of the molecule. There are five amino acid differences in the sequences of the nucleoproteins from viruses isolated in 1934 and 1968. As clone F5 could distinguish between these two proteins, one or more of the amino acids that differ were thought likely to form part of the epitope recognized. The Asn-to-His change at position 334 was shown not to play a role because a further deletion mutant of NP that lacked amino acids 255-339 was recognized efficiently by clone F5 (data not shown). Peptides covering the remaining sequence were then tested for their ability to sensitize target cells in the ⁵¹Cr release assay, and to stimulate proliferation of the CTL clone in the presence of IL-2.

Figure 1 shows the results in the ⁵¹Cr release assay. The three panels at the upper left demonstrate the specificity of clone F5 for the 1968 influenza nucleoprotein; the remaining panels show the level of lysis of the L/D^b target cell in the presence of the indicated peptides. The results showed that recognition by CTL clone F5 could be narrowed down to a 16 amino acid segment represented by peptide 365-380 derived from the 1968 sequence.

The upper half of Figure 2 shows that recognition of peptide 365-380 (1968) by clone F5 was class I MHC restricted. The lower half of Figure 2 demonstrates the effect of titrating out the concentration of peptide 365-380 (1968) while maintaining a constant ratio of CTL clone to target

Figure 1. Recognition by CTL Clone F5 (D^b Restricted) of L/D^b Target Cells in the Presence of Peptides Corresponding to Regions of the NP Amino Acid Sequence

At the top left are the three controls: L/D^b (L cells transfected with the class I MHC gene D^b) in the absence of peptide, NP1 (L cells cotransfected with D^b and the 1968 gene for NP), and L/D^b infected with X31 virus, which expresses the 1934 gene for NP. The remaining panels show the recognition of L/D^b cells in the presence of the indicated peptides at a final concentration of 35 μg/ml (19.8–21.5 μM). The ⁵¹Cr release assay was performed by testing CTL at a variety of killer:target (K:T) ratios as described in Experimental Procedures, method 1.

cells of 2:1. The optimum level of lysis was achieved with a concentration of this preparation of peptide in the assay of between 5×10^{-6} and 10×10^{-6} moles per liter.

Clone F5 also was induced to proliferate, in the presence of IL-2 containing medium and uninfected feeder cells, by peptide 365-380 (1968). Table 2 shows that this peptide induced maximal proliferation whereas peptides 345-360 and 369-382 (1968) had no effect. The result is noteworthy because the optimum concentration of peptide 365-380 (1968) required to induce proliferation by clone F5 was the same as that which induced the plateau level of cytotoxic activity in the ⁵¹Cr release assay (Figure 2).

In control experiments clone F5 was tested either by ⁵¹Cr release or by thymidine incorporation with a variety

Figure 2. Recognition of Peptide Is MHC Restricted and Concentration Dependent

(Top) Recognition by CTL clone F5 of peptide 365–380 (1968) is class I MHC restricted. Recognition of L cells transfected with the class I gene D^b is shown at the left, recognition of untransfected L cells at the right. Target cells were (a) infected with E61-13-H17 virus, which expresses the 1968 NP gene; (b) uninfected; (c) resuspended in peptide 365–380 (1968) at 20 μg/ml (11.5 μM); and (d) resuspended in peptide 369–382 (1968) at 20 μg/ml (12.3 μM).
(Bottom) Recognition by CTL clone F5 of L/D^b cells in the presence of decreasing concentrations of peptide 365–380 (1968). The ⁵¹Cr release assays were done using method 1 and a killer:target ratio of 2:1.

Table 2. Proliferation of Clone F5 with Peptide 365–380 (1968)

Peptide/Virus	Final Concentration (μg/ml)	cpm	SEM
Experiment 1			
E61-13-H17 infected		66,479	1,974
X31 infected		3,655	424
No peptide, uninfected		4,155	38
365–380 (1968)	10 (5.8 μM)	66,093	2,102
365–380 (1968)	1 (0.58 μM)	6,464	431
345–360 (1968)	10 (5.7 μM)	6,301	265
345–360 (1968)	1 (0.57 μM)	5,552	429
Experiment 2			
E61-13-H17 infected		57,391	4,918
No peptide, uninfected		1,045	68
365–380 (1968)	80 (46 μM)	47,431	1,712
365–380 (1968)	40 (23 μM)	69,671	961
365–380 (1968)	20 (11.5 μM)	89,012	2,491
365–380 (1968)	10 (5.8 μM)	83,888	544
365–380 (1968)	5 (2.9 μM)	64,689	497
369–382 (1966)	80 (49.3 μM)	1,210	92
369–382 (1968)	40 (24.6 μM)	1,195	123
369–382 (1968)	20 (12.3 μM)	1,240	213
369–382 (1968)	10 (6.2 μM)	1,212	82
369–382 (1968)	5 (3.1 μM)	1,095	123

The thymidine incorporation assay was performed as described in Experimental Procedures.

of other peptides derived from the NP sequence, but without any effect (these negative results are summarized in Table 3).

The Optimum Length of Peptide Recognized by Clone F5

In Figures 1 and 2 we have shown that clone F5 recognized the 16 amino acid peptide representing the sequence between residues 365 and 380 of the 1968 nucleoprotein. In later experiments we investigated the effect of reducing the length of this peptide.

A series was made of new peptides of decreasing lengths (during one synthesis), covering the sequences 365–379, 366–379, 367–379, 368–379, and 369–379. These peptides were then compared over a wide range of concentrations in the ⁵¹Cr release assay for their ability to induce lysis of L/D^b and EL4 (H-2b) target cells by clone F5. The experiments were performed using method 1 with the peptides present throughout the assay. The results with EL4 target cells are shown in Figure 3. On the y-axis is shown the level of lysis induced and on the x-axis the molar concentrations of the various peptides used. The

L/D^b target cells gave very similar dose-response curves (data not shown).

The most efficient peptide was 366–379 (labeled A in Figure 3). The concentration of this peptide required to induce 20% lysis of the target cells was 6.25 × 10⁻⁹ moles per liter. The negative control was 365–380 (1934 sequence), which had no detectable effect up to 2.81 × 10⁻⁴ moles per liter (G in Figure 3). An approximately 10-fold greater concentration of the longest peptide of the set, 365–379, was required to induce the same level of lysis as 366–379 (B in Figure 3). The remaining three peptides of the set, 367–379 (C), 368–379 (D), and 369–379 (E), demonstrated a requirement for an approximately 10-fold increase in peptide concentration for each reduction in length by one amino acid to achieve 20% target cell lysis. According to this criterion peptide 369–379 was at least 10⁵-fold less efficient than 366–379 at inducing lysis of EL4 target cells by clone F5 (compare curves A and E in Figure 3).

The set of peptides covering the sequence 365–379 was made during one synthesis on an Applied Biosystems peptide synthesizer. Consequently, each of the products was similar in purity, as assessed by HPLC (approximately 80%; data not shown). The relationships between the dose-response curves for each peptide shown in Figure 3 therefore can be regarded as accurate. The relatively high concentration of peptide 365–380 (1968) required for lysis (Figure 2) and proliferation (Table 2) is related to the fact that this preparation was made separately, using a manual technique, and was correspondingly less pure.

Finally, although 369–379 had a specific effect at concentrations up to 5.12 × 10⁻⁴ moles per liter (E in Figure

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Table 3. Summary of CTL Recognition of Nucleoprotein Peptides

Sequence	Date	Mol. Wt.	CTL Recognition		
			Mouse Clone F5	Mouse Clone A3.1	Human Polyclonal MG
325-339[a]	1934	1706	-	NT	-
335-349	1968	1679	-	-	+++
335-349[a]	1934	1651	-	NT	++++
345-360	1968	1785	-	-	-
345-360[a]	1934	1853	-	-	-
357-370[a]	Shared	1516	-	-	+
385-379	1968	1608	++++	NT	NT
386-379	1968	1495	+++++	NT	NT
387-379	1968	1424	+++	NT	NT
368-379	1968	1237	++	NT	NT
369-379	1968	1223	+	NT	NT
365-380[a]	1968	1736	+++	-	-
365-380	1934	1780	-	+++++	-
369-382	1968	1621	-	-	NT
369-382	1934	1685	-	+/-	NT
385-401[a]	1934	1980	-	NT	-
388-411[a]	1934	1458	-	NT	-
410-425[a]	1934	1877	-	NT	+
413-427[a]	1934	1734	-	NT	-
440-455[a]	1934	1845	-	NT	-
448-460[a]	1934	1737	-	NT	-
456-470[a]	1994	1695	-	NT	-
473-487[a]	1934	1826	-	NT	-

[a] These peptides were synthesized manually.
Peptides not synthesized manually were made on an Applied Biosystems peptide synthesizer (see Experimental Procedures). Responses are graded positive (+) or negative (-). NT: not tested.

3), the overlapping peptide 369-382 had only a barely detectable effect (F in Figure 3). The latter peptide was made in a different synthesis but was similar in purity to 369-379.

CTL Clones A3.1, B4, and B8

These three CTL clones were derived from C57BL (H-2b) mice primed and restimulated with the recombinant A virus X31, which contains the NP gene from a 1934 virus isolate (Baez et al., 1980). A3.1 has been described previously (Townsend and Skehel, 1982; Townsend et al., 1983). It also expressed the Lyt2, Thy1 phenotype and had the exact reciprocal specificity to clone F5 described above. A3.1 recognized all A viruses isolated between 1934 and 1943 but not viruses isolated between 1946 and 1979, and experiments with recombinant A viruses showed it to be specific for the 1934 influenza NP (Townsend et al., 1984b). Clones B4 and B8, derived in a separate cloning experiment from A3.1, have been shown to have the same specificity (Wraith, unpublished results).

The reciprocal relationship of the specificities of clones F5 and A3.1 for natural virus isolates led us to test the 365-380 peptide derived from the 1934 NP sequence (see Table 1) for recognition by clones A3.1, B4, and B8. The results for A3.1 in the ^{51}Cr release assay are shown in Figure 4. A3.1 recognized 365-380 (1934) but not 365-380 (1968). In addition A3.1 proliferated in response to 365-380 (1934) but not to 365-380 (1968) (data not shown). Clones B4 and B8 gave the same pattern of activ-

ity as clone A3.1. All of these clones were shown to be restricted through D^b for recognition of peptide 365-380 (1934) (data not shown).

The Effect of Preincubating the Target Cell or the Effector Cell with Peptide Antigens

All of the results described above were from experiments using method 1, where the peptide is present in solution in contact with both CTL and target cells throughout the 6 hr period of the ^{51}Cr release assay.

In Figure 5 the effect of preincubating the target cell is compared with the effect of preincubating clone F5 with peptide 365-380 (1968). Target cells treated in this way were recognized as efficiently as when peptide was present throughout the assay. Figure 5A also shows that CTL recognition of target cells prepulsed with peptide was MHC restricted, as untransfected L cells, which do not express D^b, were not recognized after prior exposure to the peptide.

Figure 5B demonstrates that pulsing the CTL clone with peptide 365-380 had no effect. Clone F5 was not able to recognize untreated L/D^b target cells after prior contact with peptide. Neither did pretreating the CTL clone with peptide have any effect on the recognition of infected, NP transfected (data not shown), or peptide pulsed target cells.

The CTL clone, which expresses the D^b molecule, was examined for the ability to lyse itself when the 365-380 (1968) peptide was present throughout the ^{51}Cr release

Figure 3. Recognition of EL4 (H-2b) Target Cells in the Presence of Decreasing Concentrations of Peptides

A; 366–379 (1968). B: 365–379 (1968). C: 367–379 (1968). D: 368–379 (1968). E: 369–379 (1968). F: 369–382 (1968). G: 365–380 (1934). The ⁵¹Cr release was assayed using method 1 as described in Experimental Procedures. The final concentrations of peptides present during the assay are shown.
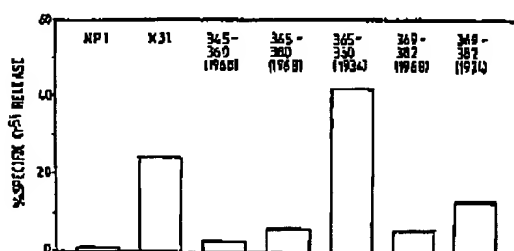
Figure 4. Recognition by CTL Clone A3.1 of Peptide 365–380 (1934)

The experiment was set up as described for Figure 1 with the peptides at a final concentration of 35 µg/ml (19.8–21.6 µM). The controls were NP1 (L cells cotransfected with Dᵇ and 1968 NP) and L/Dᵇ infected with X31 (which expresses the 1934 NP). The CTL clone was used at a killer/target ratio of 1.5:1.

Figure 5. Effects of Preincubating Target Cells or CTL Clone with Peptide

(A) Recognition by clone F5 of L/Dᵇ target cells that have been exposed to 100 µg/ml (57.8 µM) of peptide 365–380 (1968) and then extensively washed. (B) Recognition by clone F5 after it has been exposed to 100 µg/ml (57.8 µM) of peptide 365–380 and then washed. Target cells: (O) untreated L/Dᵇ cells; (●) L/Dᵇ cells exposed to peptide 365–380 (1968) then washed four times; (□) untreated L cells; (■) L cells exposed to peptide 365–380 (1968) then washed four times.

assay. F5 was labeled with ⁵¹Cr and incubated for 6 hr in medium containing peptide 365–380 (1968) at between 5 µg/ml and 100 µg/ml (2.9–57.8 µM). No self-lysis occurred (data not shown). Taken with the fact that clone F5 proliferates more efficiently when stimulated with the optimum concentration of peptide than when stimulated with virus infected feeder cells (Table 2), it is apparent that the CTL clone must have a means of avoiding autolysis.

Identification of Nucleoprotein Peptides Recognized by Human CTL

A proportion of humans primed by natural influenza infection can generate CTL in vitro that recognize the viral NP (McMichael et al., 1986). Polyclonal influenza A virus specific CTL prepared from blood donor MG were found to include a major component that was specific for NP. These were then tested on the peptides from NP presently available (summarized in Table 3).

Figure 6 shows the effect of incubating MG CTL with syngeneic target cells in the presence of each peptide during the 6 hr ⁵¹Cr release assay. The ⁵¹Cr-labeled target cells were lysed in the presence of peptide 335–349 (1934) as efficiently as after influenza A virus infection. A

lower level of target cell lysis was induced by peptides 357–370 and 410–425 (1934). It is important to note that MG CTL did not recognize peptides 365–380 from 1934 or 1968.

We have since found that MG CTL which recognize 335–349 (1934) cross-react on the homologous peptide from the 1968 sequence that differs by a Lys-to-Arg change at position 348 (Table 1). This specificity represents the characteristic cross-reactivity displayed by the majority of influenza specific CTL in man (data not shown).

Subsequent experiments showed that optimum conditions for detecting recognition of peptides by MG CTL in the ⁵¹Cr release assay could be obtained by preincubating labeled target cells with peptide (method 2 in Experimental Procedures). The remaining experiments were done in this way.

Human CTL Specific for Peptide 335–349 Are Class I MHC Restricted

To identify the HLA molecules that restricted the recognition of peptide 335–349 (1934) by MG CTL a series of target cells from different blood donors was prepared. The results are shown in Figure 7. MG CTL recognized in-

Figure 6. Screening of Nucleoprotein Peptides in the 51Cr Release Assay with Influenza A Virus Specific Polyclonal Human CTL from Donor MG

At the top the peptides listed in Table 3 are schematically represented. Beneath them is shown the level of target cell lysis that occurred with each peptide at a final concentration of 40 µg/ml (20–27 µM) in the assay. The control target cells were (A) infected with X31 virus and (O) uninfected in the absence of peptide. The 51Cr release assay was performed using method 1 and a killer:target ratio of 50:1 (see Experimental Procedures). The target cells were syngeneic PHA activated lymphoblasts.

Figure 7. Recognition of Peptide 335–349 by the Human CTL is Class I HLA Restricted

Two experiments are shown (upper and lower parts of the figure). MG polyclonal CTL were tested on PHA activated lymphoblast target cells prepared from a series of related and unrelated individuals. Results are shown as percentage of specific lysis (y-axis) at two killer:target cell ratios. Target cells: (■) uninfected; (●) infected with influenza X31 virus; and (O) uninfected and preincubated for 1 hr with peptide 335–349 at 100 µg/ml (method 2 in Experimental Procedures). The HLA A, B, and DR types of MG are shown at the top. The HLA antigens that each target cell shared with MG are shown in parentheses in each panel.

The full HLA types of each target cell were as follows: MG: A1, A30, B13, B37, C6, DR7. FG: A25, A29, B44, C5, DR1, DR7. LG: A1, A29, B44, B37, C6, DR not tested. EG: A25, A30, B44, B13, C5, DR not tested. NR: A1, A3, B8, B50, C3, C7, DR2, DR3. SE: A3, A30, B14, B18, C5, C8, DR3, DR7. PC: A3, A24, B62, B37, C3, C6, DR6. JJ: A2, A11, B35, B50, C4, C6, DR1, DR7.

fluenza A Virus infected cells that shared HLA A1, B37, or B13. Infected target cells that shared HLA A30 or class II molecules (homozygous DR7) were not recognized. Target cells treated with peptide 335–349 (1934) were recognized only when they shared the class I allele HLA B37 with MG. This showed that peptide 335–349 (1934) was recognized by MG CTL only in association with one of his four available allelic class I gene products, whereas live virus infection resulted in the expression of additional determinants on the target cell that were recognized in association with A1 and to a lesser extent with B13. The same results were obtained when either Epstein Barr virus transformed lymphoblastoid cells or PHA stimulated blast cells were used as targets in the 51Cr release assay (data not shown).

As a final specificity control 51Cr-labeled MG cells pretreated with peptide 335–349 (1934) were tested as targets for influenza A specific CTL from an unrelated donor (JJ) who shared only the HLA C6 class I allele with MG. CTL from donor JJ also were able to recognize NP expressed by recombinant vaccinia (data not shown). MG target cells pretreated with peptide 335–349 (1934) were not recognized by JJ CTL. In addition, JJ CTL did not recognize peptide 335–349 (1934) on syngeneic target cells (data not shown).

Human CTL Specific for Peptide 335–349 (1934) Are Inhibited by Monoclonal Antibodies to CD8

Lysis of influenza A virus infected or peptide treated target cells by MG CTL was measured in the presence of saturating concentrations of a variety of monoclonal antibodies known to interfere with T cell recognition or lysis. Figure 8 shows that lysis of peptide treated target cells was inhibited by antibodies to HLA B and C antigens, CD8, CD3, and the alpha chain of LFA-1. No inhibition was obtained

with antibodies to CD4 or shared determinants of HLA class II molecules. The results with virus infected target cells were similar. These results are typical of class I MHC restricted lysis mediated by CD8 (T8) positive T cells.

Discussion

Cytotoxic T Lymphocytes Recognize Short Synthetic Peptides

We have described Influenza A specific CTL from both man and mouse that can recognize short synthetic pep-

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Figure 6. Human CTL That Recognize Peptide 335–349 Are Inhibited by Antibodies to CD8

MG influenza specific polyclonal CTL were tested on autologous MG B lymphoblastoid cells that had been infected with influenza A/X31 virus (left) or uninfected but treated with peptide 335–349 at 100 µg/ml for 1 hr (right). Lysis was tested at a killer:target ratio of 25:1 in the absence of added antibody or in the presence of saturating concentrations of the antibodies shown. Standard errors were all less than 3%. Uninfected MG B lymphoblastoid cells that were not treated with peptide were lysed to 5.2% by the CTL. N.T.: not tested.

lides corresponding to regions of the viral NP sequence. Recognition of peptides on the target cell surface was antigen specific and triggered efficient lysis by CTL (Figures 1 and 7). It also induced resting CTL to become responsive to the growth-promoting effect of IL-2 (Table 2).

The epitope recognized by murine CTL clone F5 was studied in detail (Figures 3 and 4). Amino acid changes at positions 372 (Asp to Glu) and 373 (Ala to Thr) abolished recognition of peptide 365–380 by clone F5, but created a new epitope recognized by clone A3.1. These results show that the region of this peptide controlling T cell specificity must contain the residues at positions 372 and 373.

Variation in the length of the peptide containing residues 372 and 373 was shown to have a profound effect on the efficiency with which it could be recognized by clone F5 at the target cell surface. The optimum sequence we have been able to define is the 14 amino acids from position 366 to 379 (A in Figure 3). Variation in length at either end of this peptide was associated with negative shifts in the dose-response curve (B–F in Figure 3).

These experiments with class I restricted CTL were performed using the ⁵¹Cr release assay, which involves a 4–6 hr contact between T cell, target cell, and peptide. The results are clearly related to the wealth of data on class II restricted recognition of peptides obtained in proliferation assays. The optimum concentrations of peptides that induce class I restricted lysis and proliferation are in the same range as a variety of class II restricted responses (reviewed by Hackett et al., 1983). Effects similar to those shown in Figure 3 have been described in several examples of class II restricted recognition of peptides. These include guinea pig T cell recognition of insulin B chain peptide 1–16 (Thomas et al., 1981), a detailed analysis of recognition by murine T cell clones specific for peptide 46–61 of hen egg-white lysozyme (Allen et al., 1985), residues 110–121 of sperm whale myoglobin (Livingstone et al., submitted), and the amino terminus of murine myelin basic protein (Zamvil et al., submitted).

The discussion of these results has centered around the possible conformational and physicochemical restraints on a given immunogenic peptide required for it to interact with both the restriction element (class I or class II MHC molecule) and the T cell receptor (De Lisi and Berzofski, 1985; Heber-Katz et al., 1985). The notion of these interactions has been simplified by the concept of two spatially separate regions of immunogenic peptides, one making contact with the MHC molecule (the "aggretope") and the other with the T cell receptor (the epitope). A variety of experimental data are consistent with these ideas (Werdelin, 1982; Rock and Benacerraf, 1983; Heber-Katz et al., 1982, 1983; Babbitt et al., 1985).

In our example with peptide 365–379 it is tempting to assign the role of epitope to positions 372 and 373. Alterations in these two residues change T cell specificity, but have no apparent effect on MHC restriction (Figures 1, 2, and 4). Residues 365–368 control the efficiency with which the peptide is recognized on the target cell surface, but do not contribute to the specificity of CTL recognition (Figure 3). Residues 365–368 would thus fit the concept of an "aggretope." However, the validity of these assignments in the absence of any structural information remains uncertain.

Recognition of Peptides by CTL Is Class I MHC Restricted

The phenotypes of the CTL we have studied are characteristic of cells that recognize antigens in association with class I MHC gene products (Swain and Dutton, 1980; Dialynas et al., 1983). In each of the three examples, recognition by CTL of peptides on the target cell surface was antigen specific and restricted through a single class I MHC molecule (Figures 2 and 7). This was particularly revealing in the experiments with polyclonal human CTL in which only one of the three class I molecules recognized on virus infected cells functioned as a restriction element for peptide 335–349 (Figure 7). These observations extend previous work on class I MHC controlled immune response gene effects observed with whole live viruses (Blanden et al., 1979; Vitiello and Sherman, 1983).

The selection of only certain combinations of peptide and class I molecules for recognition by CTL is very similar to the results obtained with nonlytic T cells restricted through class II MHC molecules. Discussion of these effects has focused on the MHC molecules themselves and the T cell repertoire. Polymorphic MHC molecules may bind antigenic peptides with a hierarchy of affinities, and individuals may vary in their T cell repertoires through inherited or acquired diversity in their T cell receptor genes (reviewed by Schwartz, 1985; Shevac, 1982; Zinkernagel and Doherty, 1979; Benacerraf, 1978; Jerne, 1971).

We have shown in Figures 5 and 7 that the peptides recognized by class I restricted CTL can associate with the target cell surface. It is possible, in view of results by Babbitt et al. (1985), that the peptide 365–380 (1980) may bind the Dᵇ molecule. Both the target cell and the CTL clone express the Dᵇ molecule (data not shown). Efficient lysis of target cells under conditions where peptide is in contact with both target cell and CTL clone may be explained by

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the resistance of the clone to autolysis. However, as we have no means of detecting peptides on the cell surface other than by CTL recognition, this interpretation is speculative.

The observation that pretreating CTL clone F5 with peptide does not enable it to recognize the untreated L/Db target cell implies that peptide 365–380 (1968) does not bind to the T cell receptor in the absence of an appropriate target cell membrane.

A detailed analysis of the binding characteristics of the peptides we have studied will help to clarify these issues.

Do All Somatic Cells Bearing Class I Molecules Present Degraded Viral Proteins to CTL?

We have identified three epitopes of the NP molecule that can be replaced in vitro with synthetic peptides 11 to 18 amino acids in length. Each of the three epitopes exists between amino acids 335 and 385 of the NP sequence. These results, taken with our previous findings (see the Introduction), raise the possibility that degradation or denaturation of NP may take place in the target cell before CTL recognition occurs (Townsend et al., 1985). The alternative explanation is that this region of the molecule is accessible as a segmental or linear epitope in the folded molecule. The three-dimensional structure of NP is not available to judge. Experiments are in progress to compare native with denatured and degraded NP for their ability to sensitize target cells for lysis by CTL. In addition, we are raising antibodies to the peptides recognized by CTL. These may differentiate between native and degraded NP on the surface of transfected or virus infected target cells.

If degradation of viral proteins is required in a target cell prior to CTL recognition, questions arise about where and how this might occur. Recent work has shown that influenza specific CTL restricted through class II molecules exist in both mouse and man (Lukacher et al., 1985; Kaplan et al., 1984). Circumstantial evidence in these experiments was consistent with a processing step by the target cell bearing class II molecules. For instance, presentation of purified NP by lymphoblastoid cell lines to human class II restricted CTL was inhibited by chloroquine (Fleischer et al., 1985).

We have not been able to detect any effect of chloroquine on the recognition by class I restricted murine CTL (which are known to recognize peptide 365–380) of virus infected or NP transfected target cells (Gotch, unpublished work). Initial results with class I restricted human CTL show that NP purified from influenza virus particles is not capable of sensitizing lymphoblastoid target cells for lysis by MG CTL (data not shown).

If a proteolytic system does exist that is involved in presentation of antigen to class I restricted CTL, it should be present in all the cell types capable of being recognized by CTL, act on newly synthesized viral proteins, and be resistant to the effects of chloroquine and other agents thought to inhibit lysosomal degradation of proteins.

In conclusion, we have demonstrated that the epitopes of influenza NP recognized by class I restricted CTL on the surface of their target cells can be defined with short synthetic peptides. Preliminary evidence suggests that the peptides recognized by CTL can associate with the target cell membrane. The results are consistent with the suggestion that degradation of NP may be required in the target cell prior to CTL recognition. All of the phenomena we have described with class I restricted CTL are closely related to well established findings with class II restricted T cells.

Experimental Procedures

Murine CTL Clones
Clones F5 and A1.1 were isolated from C57BL/6 (obtained from OLAC) or C57BL (obtained from NIMR Mill Hill) by limiting dilution as described previously (Townsend et al., 1983, 1985). They were maintained in vitro by stimulation with A virus infected syngeneic spleen cells in TCGF conditioned medium exactly as described previously (Townsend and Skehel, 1984). For some experiments clone F5 was maintained with uninfected feeder cells and TCGF conditioned medium containing peptide 365–380 (1968) at a final concentration of 10 µg/ml. The optimum growth-promoting activity of peptide was assessed by prior titration in a thymidine incorporation assay.

Induction and Testing of Human Polyclonal CTL
The method used was based on that described previously (McMichael and Askonas, 1978) with some modifications (McMichael et al., 1986). Peripheral blood lymphocytes (PBL) were incubated in RPMI 1640 medium (Gibco) in the absence of serum with influenza X31 virus for 1 hr at 37°C. Fetal calf serum was added to 5% and the flask incubated for a further 7 days. The cultures were then harvested and resuspended in RPMI 1640, 10% FCS for use in the ⁵¹Cr release assay.

Target cells were prepared and chromium labeled as previously described (McMichael and Askonas, 1978; McMichael et al., 1986). They were either autologous Epstein Barr virus transformed B lymphoblastoid cell lines or PBL activated for 3 days with phytohemagglutinin at 2 µg/ml. For the peptide experiments, methods 1 and 2 described for the mouse CTL experiments were used.

⁵¹Chromium Release Assay
A standard procedure was used (Zweerink et al., 1977; Townsend et al., 1983, 1984; McMichael and Askonas, 1978) with the following modifications for testing peptides.

Method 1
Peptides were made up in RPMI medium containing 10% FCS and 10 mM Hepes buffer at pH 7.4 (RPMI/10) at 4 mg/ml. This stock solution was then diluted in RPMI/10 to four times the final concentration required in the assay and dispensed in 0.05 ml aliquots to experimental and control wells of 96 well microtiter trays. Target cells were harvested, resuspended in 0.5 ml RPMI/10 containing 100 µCi of ⁵¹Cr, incubated at 37°C for 90 min, then washed three times in 10 ml PBS, and once in RPMI/10, and resuspended in RPMI/10. Labeled target cells (1 × 10⁴ or 2 × 10⁴) in 0.05 ml RPMI/10 were added to round or flat-bottomed wells of 96 well microtiter trays containing 0.05 ml aliquots of peptides. Additions to this mixture were as follows: to experimental wells, CTL in 0.1 ml to make up the killer:target (K/T) ratios shown in the figures; to control wells, 0.1 ml of RPMI/10; and to total release wells, 0.1 ml of 5% Triton-X 100. The assay was then performed as previously described.

Method 2
Target cells were harvested, washed twice in serum free RPMI, then resuspended in 0.4 ml serum free RPMI containing 100 µCi ⁵¹Cr and peptide at a final concentration of 100 µg/ml (approximately 80 µM). The mixture was then incubated for 90 min at 37°C, washed three times in 10 ml PBS, washed once in 5 ml RPMI/10, and resuspended in RPMI/10. Labeled target cells (1 × 10⁴ or 2 × 10⁴) were then dispensed in 0.1 ml aliquots in microtiter trays. The assay was then continued as previously described. A minor modification was used in some of the experiments with human CTL. Target cells were labeled with ⁵¹Cr for 90 min in the absence of peptide, washed twice in serum free RPMI, then resuspended in 0.4 ml serum free RPMI containing peptide at 100 µg/ml for 1 hr at 37°C. The cells were then washed three times and dispensed in microtiter trays. The assay was then con-

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tinued as previously described. These two variations of method 2 gave identical results.

Percentage of specific ^{51}Cr release was calculated as follows:

$$\frac{(\text{Release by CTL} - \text{medium release})}{(2.5\% \text{ Triton release} - \text{medium release})} \times 100$$

Each experimental point was measured in duplicate against quadruplicate controls in medium alone. Spontaneous ^{51}Cr release by targets in medium alone varied between 10% and 17% for murine target cells and between 15% and 28% for human target cells of counts released by Triton.

Dose-Response Titrations of Peptides

For the dose-response curves in Figure 3, EL4 (H-2b) target cells were used. Peptides were dispensed in round-bottomed 96 well sterilin microtiter plates as described above in 0.06 ml, and a series of nine doubling dilutions made over a dose range optimized by trial and error in previous experiments. ^{51}Cr-labeled EL4 cells (10^4) in 0.06 ml were added to each well followed by 1.5×10^4 CTL clone F5 in 0.1 ml. The assay was then performed as previously described. The results in Figure 3 were calculated in the following way to normalize the titrations to the 0% line:

$$\frac{(\text{Release in presence of CTL and peptide} - \text{release by CTL alone})}{(2.5\% \text{ Triton release} - \text{release by CTL alone})} \times 100$$

The spontaneous release of ^{51}Cr from target cells in the presence of CTL but no peptide was 14% of the counts released by Triton. Within the dose ranges shown in Figure 3 none of the peptides alone had any detectable effect on the spontaneous release of ^{51}Cr from target cells.

The Thymidine Incorporation Assay

CTL clone F5 was fed with virus infected syngeneic spleen cells and TCGF conditioned medium as previously described (Townsend et al., 1983; Townsend and Skehel, 1984). Three to four days later the responding cells were split 1:2 and fed with fresh TCGF conditioned medium. This procedure was repeated every 3 to 4 days for at least 14 days. By this time the CTL clone stopped dividing, even when fed with fresh conditioned medium. Further cell division was absolutely antigen dependent.

Peptide dilutions were prepared from a stock solution at 4 mg/ml in RPMI/10 that had been filter sterilized. Diluted peptides or medium alone were then dispensed in 0.06 ml triplicate aliquots to wells of round-bottomed 96 well sterilin microtiter plates. To these aliquots was added 0.15 ml RPMI/10 containing 10^4 clone F5 cells; 5×10^5 uninfected or E61-13-H17 infected, 2000 rad irradiated syngeneic spleen cells; and 0.05 ml of a Con A stimulated rat spleen cell supernatant (Townsend and Skehel, 1984). The cultures were incubated for 3 days at 37°C in 5% CO₂ and pulsed with 1 μCi of ^{3}H thymidine for 8 hr; the cells were then harvested on a Titertek 560 cell harvester. The ^{3}H thymidine incorporation by the CTL clone was measured by scintillation counting. Triplicate wells were set up for each peptide dilution, and the mean and standard error of the mean were calculated for each point.

Peptide Synthesis

Some peptides in Table 3 (see footnote a) were synthesized manually by Merrifield's solid-phase method (Merrifield, 1963) using t-Boc suitably protected amino acids and the appropriately derivatized amino acid resin (Sigma). N,N'-dicyclohexylcarbodiimide (DCC) was used as the coupling agent. The peptide was cleaved off the resin with hydrogen bromide and trifluoroacetic acid, then desalted on G15 Sephadex with 50% acetic acid. When arginine and cysteine were present the peptides were further deprotected in sodium and liquid ammonia, then desalted. The crude freeze dried material was analyzed on a Beckman 121B amino acid analyzer, and by high pressure liquid chromatography (HPLC).

The remaining peptides in Table 3 were synthesized by solid phase techniques on an Applied Biosystems peptide synthesizer, model 430A. Commercially available amino acid phenylacetamidomethyl resins and t-Boc suitably protected amino acids were used. All

couplings were performed using a 2.5 molar excess of t-Boc amino acid and DCC over the number of milliequivalents of amino acid on the resin. In the case of Asn and Gln a 2.5 molar excess of the amino acid, DCC, and N-hydroxytriazole was used. The peptides were deprotected and removed from the resin simultaneously by treatment with anhydrous hydrogen fluoride in the presence of anisole, dimethyl sulfide, and indole. The peptides were separated from the various organic side products by extraction with ether and isolated from the resin by extraction with 5% acetic acid and subsequent lyophilization. The purity of the crude product was determined by HPLC on a C18 reverse phase column. All the peptides synthesized by this method contained > 90% of the desired product.

Influenza Virus Strains

The recombinant A viruses X31 (Baez et al., 1980) and E61-13-H17 (Lubeck et al., 1979, where this virus is referred to as recombinant 33; P. Palese, personal communication) differ only in the origin of their genes for nucleoprotein. E61-13-H17 contains the 1968 NP gene from A/HK/8/68, and X31 contains the 1934 NP gene from A/PR/8/34. Virus was grown in the allantoic sacs of 11 day old embryonated chicken eggs, and stored as infectious allantoic fluid at -70°C.

Human Blood Donors

All donors were healthy volunteers. EG and LG were daughters of MG and FG. JJ, NR, PC, and SE were unrelated. HLA types were determined by the standard technique of the National Institutes of Health.

Inhibition of Human CTL with Monoclonal Antibodies

The monoclonal antibodies used were as follows: UCHT1, anti-CD3, a gift from Dr. P. C. L. Beverley, University College, London; anti-Leu 3a, anti-CD4, a gift from Dr. R. Evans, Memorial Sloan-Kettering Institute, New York; B9A1, anti-CD8, a gift from Dr. C. Mawas, Centre d'immunologie, Marseille; SG171, anti-HLA class II, a gift from Dr. S. Goyert, Hospital for Joint Diseases, New York; PA2.6 anti-HLA class I, a gift from Dr. P. Parham, Stanford University; MHM5 anti-HLA B and C; and MHM24 anti-LFA-1α chain. Antibodies were added to the mixture of killer and target cells at the initiation of the chromium release assay to a concentration of 1/200 ascites. This was determined to be in excess of the concentration required to saturate all the antigenic sites of the cells in the assay.

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Localization of Epstein-Barr Virus Cytotoxic T Cell Epitopes Using Recombinant Vaccinia: Implications for Vaccine Development

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Summary

There is considerable interest in designing an effective vaccine to the ubiquitous Epstein-Barr virus (EBV). An important role for EBV-specific cytotoxic T lymphocytes (CTLs) in eliminating virus-infected cells is well established. Limited studies using a small number of immune donors have defined target epitopes within the latent antigens of EBV. The present study provides an extensive analysis of the distribution of class I–restricted CTL epitopes within EBV-encoded proteins. Using recombinant vaccinia encoding individual EBV latent antigens (Epstein-Barr nuclear antigen [EBNA] 1, 2, 3A, 3B, 3C, LP, and LMP 1), we have successfully localized target epitopes recognized by CTL clones from a panel of 14 EBV-immune donors. Of the 20 CTL epitopes localized, five were defined at the peptide level. Although CTL clones specific for nine epitopes recognized both type 1 and type 2 transformants, a significant number of epitopes (7/16 epitopes for which EBV type specificity was determined) were detected only on type 1 EBV transformants. Vaccinia recombinants encoding EBNA 3A and EBNA 3C were recognized more frequently than any other vaccinia recombinants used in this study, while no CTL epitopes were localized in EBNA 1. Surprisingly, epitope specificity for a large number of EBV-specific CTL clones could not be localized, although vaccinia recombinants used in this study encoded most of the latent antigens of EBV. These results suggest that any EBV vaccine based on CTL epitopes designed to provide widespread protection will need to include not only latent antigen sequences but also other regions of the genome. The apparent inability of human CTLs to recognize EBNA 1 as a target antigen, often the only latent antigen expressed in Burkitt's lymphoma and nasopharyngeal carcinoma, suggests that EBV-specific CTL control of these tumors will not be feasible unless the down-regulation of latent antigens can be reversed.

Protective immunity to viral infection requires the development of memory T cells that recognize viral antigens in association with class I MHC. Earlier studies on influenza virus first highlighted the important role of CD8⁺ CTLs, which recognize virus-encoded proteins in the form of short peptides (1). Since it is well established that immunization with whole viral proteins is unable to elicit an efficient CTL response, interest has been directed towards peptide vaccines based on defined epitope sequences. This is particularly the case with oncogenic viruses, since individual viral genes introduced in recombinant vectors have the potential to initiate tumorigenic processes. Thus, it is important to determine the distribution of these epitopes within viral proteins and the frequency with which infected cells from a significant cohort of immune donors present these epitopes in association with MHC class I alleles. Because of the potential importance of CTL epitopes in the future development of a vaccine to EBV, a herpes virus with known oncogenic potential, there is considerable interest in defining these EBV-encoded molecules recognized by CTLs.

EBV is the etiological agent of infectious mononucleosis (IM)¹ and is associated with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (2), lymphomas in immunocompromised individuals (3), and more recent evidence suggests an association with Hodgkin's lymphoma (4). Tw

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¹ Abbreviations used in this paper: BL, Burkitt's lymphoma; EBNA, Epstein-Barr nuclear antigen; IM, infectious mononucleosis; LCL, lymphoblastoid cell line; LMP, latent membrane protein; LP, leader protein; NPC, nasopharyngeal carcinoma; TP, terminal protein; UM, unfractionated mononuclear.

types of EBV (1 and 2, also referred to as A and B) are recognized that show DNA sequence divergence within the BamHI WYH and E regions of the genome (5–7). In vitro, the virus transforms human B cells into lymphoblastoid cell lines (LCLs), which express a limited number of viral gene products, including a family of Epstein-Barr nuclear antigens (EBNA): 1, 2, 3A, 3B, and 3C, leader protein (EBNA LP), latent membrane proteins (LMP 1 and 2), and terminal proteins (TP 1 and 2) (5). An alternative nomenclature also in current use designates the EBNA family as EBNA 1, 2, 3, 4, and 6, and EBNA-5/LP (4, 5). In contrast, latent antigen expression in BL and NPC is restricted to EBNA 1 (in some instances LMP is also expressed in NPC) (8).

In all previously infected individuals, the virus persists for life as a latent infection in B cells and is apparently restrained by a population of EBV-specific CTLs (9, 10). This CTL response is a classic virus-specific response (CD8, class I restricted) (10), though CD4 class II–restricted cells have also been described (9). The key observation in defining the first peptide epitope recognized by EBV-specific CTLs was that in certain donors it was possible to exploit the allelic polymorphism in the EBNA proteins between type 1 and type 2 EBV by isolating type 1–specific CTL clones (11). These clones provided an opportunity to screen selected EBNA peptides for reactivity on type 2 transformants. This led to the definition of an EBV-specific CTL epitope that was present on type 1 but not type 2 transformants (12). A second epitope, derived from EBNA 3C and present on both type 1 and type 2 transformants, has also been described (13). While this approach was very successful in defining a limited number of CTL epitopes, their overall distribution within EBV latent antigens was largely undetermined.

Two recent technical advances from our laboratories have facilitated this study. First, the construction of recombinant vaccinia capable of expressing individual EBV latent antigens, and second, the establishment of an EBV-negative host cell (anti-μ B cell blasts) for these recombinant vaccinia (14, 15). In the present report, we have localized EBV CTL epitopes recognized by multiple CTL clones from a panel of immune donors to generate the first comprehensive analysis of the distribution of CTL epitopes within the EBV latent antigens. This approach was combined with peptide epitope mapping, which permitted the identification of a number of new CTL epitopes. Moreover, since EBV infection is associated with BL and NPC, another important objective of this study was to determine whether any CTL epitopes are localized within EBNA 1 and/or LMP.

Materials and Methods

Establishment and Maintenance of EBV-transformed Cell Lines. LCLs were established from a panel of healthy EBV-seropositive donors listed in Table 1 by exogenous virus transformation of peripheral B cells using type 1 (B95.8 and IARC-BL74) or type 2 (Ag876) EBV isolates (11), and were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin plus 10% FCS (growth medium). LCLs were desig-

nated with the donors initial followed by the transforming virus source (e.g., LC/B95.8 designates B lymphocytes from donor LC transformed with virus from the B95.8 cell line).

Generation of Anti-μ B Cell Blasts. Unfractionated mononuclear (UM) cells were separated on Ficoll/Paque (Pharmacia, Uppsala, Sweden) and depleted of T cells using E-rosetting (16). The enriched B lymphocytes were cultured in growth medium containing anti-IgM (μ chain specific) coupled to acrylamide beads (Bio-Rad Laboratories, Richmond, CA), human rIL-4 (50 U/ml; Genzyme, Boston, MA), and highly purified human rIL-2 from *Escherichia coli* (20–40 U/ml) (17, 18). After 48–72 h, B cell blasts were suspended in growth medium supplemented with rIL-2 (20–40 U/ml). The B cells continue to divide two to three times/wk for 3 wk in the presence of rIL-2. These cells are referred to as anti-μ B cell blasts.

Vaccinia Virus Recombinants. Recombinant vaccinia constructs for different EBNA genes have been previously described (14, 15, 19). All EBV sequences were derived from the B95.8 strain of virus. The EBNA 3A sequence was derived from the cDNA Clone T216 (7), which consists of the 5' portion of the EBNA 3A coding sequence, crossing the splice site and extending to the EcoRI site located at position 93166. This plasmid was digested with PstI and collapsed onto itself to remove 5' noncoding sequences. The resulting plasmid, T216P, was opened at the EcoRI site and ligated to the genomic EcoK fragment derived from plasmid PMH36, recreating the intact full-length EBNA 3A coding sequence. The EBNA 3A coding region was excised with PstI and EcoRV and ligated to PscI1 at the SmaI site. The EBNA 3B was derived from the cDNA clone PMLPT7. The portion of the open reading frame crossing the splice junction was excised with SpeI and XbaI and inserted into SpeI-digested pBluescript. The resulting plasmid was digested with SpeI and EcoRI and ligated to a 440-bp genomic fragment from EcoRI (95243) to SpeI (95683). The resulting plasmid (pBS:E3B) was digested with EcoRI and XbaI to release full-length coding region of EBNA 3B and ligated to the SmaI site of PSC11.

All constructs had the potential to encode the relevant full-length EBV protein except for EBNA 2 deletion mutants. The diagrammatic representation of EBNA 2 deletion mutants is shown in Fig. 1. All constructs utilize the authentic start and stop codons. All constructs are under the control of vaccinia virus P7.5 promotor, except EBNA 1, which has been described elsewhere (19). A vaccinia virus construct made from insertion of the pSC11 vector alone and negative for thymidine kinase (VaccTK⁻) was used as control.

Source of Generation of EBV-specific CTL Clones. UM cells (10^6/ml) from each donor were cultivated with irradiated (8,000 rad) autologous type 1 (B95.8) LCLs (responder to stimulator ratio of 200:1) in 2-ml culture wells (Linbro Chemical Co., Hamden, CT) for 3 d in growth medium. In the case of donors LC and IM, CTL clones were also established after stimulation with BL74-transformed autologous LCLs. CTL clones generated by seeding in 0.35% agarose were established from these donors and maintained as described earlier (9, 11). Colonies were harvested after 3 d and amplified in culture with biweekly restimulation with rIL-2 and autologous LCL.

Cytotoxicity Assay on LCLs. CTL clones from each donor were screened in a standard 5-h ^{51}Cr release assay (at an E/T ratio of 5:1 or 10:1) for specific reactivity against autologous types 1 and 2 and allogeneic type 1 LCLs as previously described (11). Clones were designated as being EBV-specific on the basis of recognition of the autologous type 1 LCL and lack of recognition of MHC-unrelated LCLs and autologous anti-μ B cell blasts and/or PHA blasts.

Cytotoxicity Assay on Recombinant Vaccinia Virus–infected Targets.
Anti-μ B cell blasts or type 2 LCLs were infected with recombinant
vaccinia viruses at a multiplicity of infection (M.O.I.) of 10:1 for
1 h at 37°C as described earlier (14). After 14–16 h, cells were washed
with RPMI 1640 and incubated with ^{51}Cr for 90 min, washed
three times, and used as targets in a standard 5-h ^{51}Cr-release assay
as described above. The effector cells were added to the assay at
E/T ratios between 5:1 and 10:1. To confirm the expression of EBV
antigens in anti-μ B cell blasts and/or LCLs after recombinant vac-
cinia infection, the infected cells were also processed for immuno-
blotting and immunofluorescence (14).

Screening of CTL Clones for Peptide Epitope Specificity. To iden-
tify the CTL epitopes recognized by EBV-specific CTL clones from
each donor, a series of peptides from EBNA or LMP 1 were syn-
thesized (10–15 amino acids) (20) based on the known sequence
of the B95.8 strain of EBV. Peptides selected were primarily based
on the results of recombinant vaccinia CTL assays and those that
corresponded to predicted algorithms (21, 22). Peptides were dis-
solved in RPMI 1640 and distributed into U-bottomed microdilu-
tion plates (200 μg/ml, 20 μl/well) and frozen at −70°C until
required. ^{51}Cr-labeled anti-μ B cell blasts were added to each well
(2 × 10^5/ml, 50 μl/ml) and incubated at 37°C. After 1 h, 130
μl of cloned autologous CTLs were added to the reaction mixture
(final E/T ratio as indicated), and the assay was conducted as de-
scribed above.

Results

Localization of EBV CTL Epitopes in EBV-immune Donors. A
total of 362 clones were isolated from a panel of 14 healthy
EBV-immune donors after stimulation with irradiated autol-
ogous B95.8- or BL74-transformed LCLs. Of these, 212 were
EBV-specific CTLs. The proportion of these specific CTL
clones from each donor varied from 100% (17/17 for donor
DM) to as low as 16% (8/51 for donor AS) (Table 2).

To define the antigen specificity of the 212 EBV-specific
CTL clones, autologous anti-μ B cell blasts or type 2 LCLs
were infected with recombinant vaccinia expressing individual
EBV latent antigens (and Vacc.TK⁻) and used as targets in
a ^{51}Cr release assay. The reactivity of five EBV-specific clones
from one of the 14 donors (DD) is illustrated in Fig. 2, and
demonstrates that two of these clones recognize Vacc.EBNA
3A (CTL5 and CTL13), two recognize Vacc.LPM1 (CTL8
and CTL10), while the antigen specificity of one clone (CTL9)
was not defined by the panel of vaccinia constructs. Table

Table 1. *HLA Antigen (Class I) Type of the EBV-immune Donors Included in this Study*

Donor	HLA typing
LC	A1, B8, B18
IM	A1, A11, B8, B51
DM	A24, A29, B44, B47
CM	A11, A24, B7, B44
PM	A11, A29, B7, B44
AS	A2, A24, B51, B62
NB	A2, A24, B7, B35
DD	A1, A3, B8, B40
LX	A24, B15, B38
LL	A2, B7, B44
CS	A2, A23, B35, B44
SJ	A2, A3, B7, B44
JA	A2, A11, B7, B15
JS	A1, A2, B8, B51

2 presents a summary of the EBNA/LMP 1 vaccinia con-
structs recognized by CTL clones from each of the 14 donors.
All vaccinia constructs except Vacc.EBNA 1 and Vacc.EBNA
3B were recognized by EBV-specific CTL clones. However,
it should be emphasized that recognition of Vacc.EBNA 3B-in-
fected anti-μ B cell blasts by EBV-specific CTL clones was
assessed in only five donors (Table 2). A dominant response
through a single vaccinia construct was observed with some
donors (DM, CM, and CS recognized primarily Vacc.EBNA
3C) (Table 2). An important feature of these results was that
the majority (145/212) of EBV specific clones failed to recog-
nize any of the latent antigens encoded by vaccinia constructs
(Table 2). This was particularly evident in the case of donors
PM and JA, where none of the clones recognized cells in-
fected with any of the vaccinia constructs.

*MHC Class I Restriction of Vaccinia-localized CTL Epi-
topes.* Of the 212 EBV-specific CTL clones investigated in
the present study, 20 distinct epitopes were localized using
vaccinia constructs. CTL clones specific for seven of these
epitopes were type 1 specific, while clones specific for nine

Figure 1. Diagrammatic representation of B95.8
EBNA 2 sequence and of vaccinia constructs encoding
EBNA 2 and its deletion mutants. The construct encoding
the full-length EBNA 2 is designated (■), while the
regions deleted from EBNA 2 are designated (□). De-
tails for the preparation of these mutants has been pub-
lished earlier (15). Each recombinant vaccinia had the ca-
pacity to encode truncated EBNA 2 proteins with the
following amino acid residues deleted: Vacc.HB has amino
acid residues 19–118 deleted; Vacc.BB has amino acid
residues 151–327 deleted; Vacc.St has amino acid residues
203–237 deleted; Vacc.Sp has amino acid residues 251–384
deleted; and Vacc.PP has amino acid residues 405–480
deleted.

Table 2. *Recognition of Vaccinia Recombinants Encoding EBV Latent Antigens by EBV-specific CTL Clones from Immune Donors*

Donor	No. of clones	EBV specific clones‡	Vacc. EBNA 1	Vacc. EBNA 2§	Vacc. EBNA 3A	Vacc. EBNA 3B	Vacc. EBNA 3C	Vacc. EBNA LP	Vacc. LMP 1
LC	47	44	−‖	2	7	−	−	−	1
IM	26	15	−	−	4	−	−	−	−
DM	17	17	−	−	−	NT¶	12	−	−
CM	29	19	−	−	1	NT	6	−	−
PM	25	17	−	−	−	NT	−	−	3
AS	51	8	−	−	1	NT	−	1	1
NB	42	8	−	5	1	NT	−	−	3
DD	21	17	−	−	3	−	−	−	−
LX	14	5	−	−	3	−	−	2	1
LL	19	16	−	−	1	NT	2	2	−
CS	15	12	−	−	−	NT	4	−	−
SJ	24	19	−	−	1	NT	−	−	−
JA	12	4	−	−	−	NT	−	−	−
JS	20	11	−	2	−	−	−	−	−
Total	362	212	0	9	22	0	24	3	9

* Total number of clones tested for cytolytic activity.
‡ This column summarizes the number of EBV-specific CTL clones isolated from each individual donor.
§ This column refers to the number of clones recognizing autologous anti-μ B cell blasts infected with recombinant vaccinia encoding EBNA 2.
‖ No clones reactive to vaccinia construct.
¶ Not tested.

other epitopes recognized both type 1 and type 2 transformants. The type specificity of four epitopes was undefined (Table 3). The HLA restriction of the specific epitopes was determined by comparing the lysis of autologous LCLs and allogeneic LCLs sharing one or more alleles (Table 3). CTL clones restricted through eight different alleles were observed while the restricting alleles for five EBV CTL epitopes were undetermined (Table 3). An important feature of these results was that different clones restricted through HLA A2, B7, B40, B8, and B51 each recognized epitopes included in two different latent antigens (Table 3). This observation implies that a single allele can present two distinct EBV CTL epitopes.

More precise localization of CTL epitopes within EBNA 2 was facilitated by the availability of deletion mutants encoding truncated EBNA 2 proteins. In all, nine EBNA 2-specific CTL clones restricted through three different alleles (HLA A2, B18, and B7) were isolated (Table 3). The A2-restricted clones from donors JS and NB recognized four deletion mutants (Vacc.BB, Vacc.St, Vacc.PP and Vacc.Sp) with a level of lysis comparable with Vacc.EBNA 2. In contrast, the Vacc.HB mutant, which had a deletion affecting the NH₂ terminus of EBNA 2 protein (Fig. 1), was not recognized by these clones. Data from one such A2-restricted CTL clone from donor NB is shown in Fig. 3 a. In contrast, the HLA B18-restricted EBNA 2-specific CTL clones (from donor LC), failed to recognize Vacc.Sp and Vacc.BB deletion mutants, which had overlapping deletions for amino acids 251–327 of EBNA 2 protein (Figs. 1 and 3 b).

Based on the results obtained from the vaccinia experiments, EBV peptides from respective EBNA/LMP 1 regions were screened for their ability to sensitize autologous anti-μ B cell blasts for EBV-specific CTL lysis. In some instances, this involved the selection of 15–20-mer peptides from individual

Figure 2. Specific lysis by EBV-specific CTL clones (CTL5, 8, 9, 10, and 13) from donor DD of autologous LCLs (DD/B95.8) and Vacc. EBNA 1, 2, 3A, 3B, 3C, LP, LMP 1, and TK⁻-infected DD anti-μ B cell blasts. Anti-μ B cell blasts were infected for 12–14 h (M.O.I., 10:1) with vaccinia constructs and processed for standard ⁵¹Cr release assay. Vacc.TK⁻ was used as a control recombinant vaccinia. Results are expressed as percent specific lysis observed in a standard 5-h chromium-release assay. An E/T ratio of 5:1 was used throughout the assay.

Table 3. *Summary of the Distribution of MHC Class I–restricted EBV CTL Peptide Epitopes within EBV Latent Antigens Localized by Recombinant Vaccinia*

EBV antigen recognized	HLA restriction	Peptide epitope	EBV type specificity	Donor(s)
EBNA 2	HLA A2	DTPLIPLTIF*	Type 1	JS and NB
EBNA 2	HLA B18	PRSPTVFYNIPPMPL†	Type 1	LC
EBNA 2	HLA B7	Undefined	Type 1 and 2	NB
EBNA 3A	HLA B8	FLRGRAYGL§	Type 1	LC and IM
EBNA 3A	HLA B7	Undefined	Type 1 and 2	NB and SJ
EBNA 3A	HLA A11 or A24	Undefined	Type 1	CM
EBNA 3A	HLA B51	Undefined	Type 1	AS
EBNA 3A	Undefined	Undefined	ND	LX
EBNA 3A	HLA 40	Undefined	ND	DD
EBNA 3A	Undefined	Undefined	Type 1 and 2	LL
EBNA 3C	HLA A24 or B44	RGIKEHVIQNAFRKA†	Type 1	CM and DM
EBNA 3C	HLA B44	EENLLDFVRF‖	Type 1 and 2	DM, CM, CS, and LL
EBNA 3C	Undefined	Undefined	Type 1 and 2	CS
EBNA LP	Undefined	Undefined	Type 1 and 2	NB and LL
LMP 1	HLA A2	Undefined	Type 1 and 2	LL and NB
LMP 1	HLA B51	Undefined	Type 1 and 2	AS
LMP 1	HLA B40	Undefined	ND	DD
LMP 1	HLA A24	Undefined	Type 1 and 2	AS
LMP 1	Undefined	Undefined	Type 1	LC
LMP 1	HLA B8	Undefined	ND	DD

* Reference 23.
† This study.
§ Reference 12.
‖ Reference 13.

latent antigens that corresponded to predicted algorithms (20–22). However, to define epitopes from EBNA 2. peptides were selected that corresponded to the relevant vaccinia deletion mutant. Of the 20 distinct CTL epitopes localized in this study, five were defined at the peptide level while the other 15 remained undefined (Table 3). Of these five epitopes, three have been previously published (12, 13, 23), while new epitopes from EBNA 2 and EBNA 3C are presented in Table 3. The results of experiments that define these new epitopes are included in Fig. 4, *a* and *b*. The HLA B18–restricted EBNA 2–specific clone recognized autologous anti-μ B cell blasts sensitized with peptide PRSPTVFYNIPPMPL (residue number 276–290), while the EBNA 3C–specific clone, restricted through either HLA A24 or B44, recognized peptide RGIKEHVIQNAFRKA (residue number 332–346) (Fig. 4, *a* and *b*).

Discussion

There is convincing evidence that EBV-specific memory T cells are responsible for controlling the level of EBV-positive B lymphocytes, which all healthy seropositive individuals carry for life after primary infection with the virus. Experimental support for the existence of this protective memory T cell population came from the observation that in virus-infected cultures of mononuclear lymphocytes from seropositive (but not seronegative) donors, the initial proliferation of EBNA-positive B cells was followed by a complete T cell–dependent regression of growth such that LCLs could not be established from subcultures (24, 25). This observation suggested that the latent antigens expressed by these LCLs were a source of target antigens. Indeed, we have recently demonstrated the existence of CTL epitopes within three of the EBNA proteins by screening individual clones against a panel of peptides derived from a range of latent antigens. Although these studies identified several CTL epitopes (12, 13, 23), there has been no previous attempt to determine the relative distribution of CTL epitopes within EBV proteins, recognized by healthy immune donors expressing an array of MHC class I alleles. Such an evaluation is a mandatory prerequisite for any future CTL-based vaccine to EBV. The present study provides an extensive analysis of the distribution of CTL epi-

Figure 3. Functional analysis of EBNA 2-specific CTL clones from donors NB (a) and LC (b) generated from an in vitro stimulation with autologous B95.8 LCLs and tested on autologous type 1 LCL (NB/B95.8 or LC/B95.8) and anti-μ B cell blasts infected with recombinant vaccinia carrying EBNA 2 gene deletions (Vacc.HB, BB, St, PP, and Sp), Vacc.EBNA 2, or Vacc.TK⁻. Results are expressed as in Fig. 2. For details on the amino acids deleted from each recombinant vaccinia, see legend to Fig. 1.

Figure 4. Recognition of peptide-sensitized autologous anti-μ B cell blasts by EBV-specific CTL clones from donors LC (a) and CM (b). 51Cr-labeled anti-μ B cell blasts were presensitized with each peptide for 1 h and subsequently screened in a CTL assay using the relevant CTL clone. An E/T ratio of 5:1 was used. The results are expressed as in Fig. 2.

topes within viral-encoded proteins. Using recombinant vaccinia encoding individual EBV latent antigens, we have successfully localized target epitopes recognized by CTL clones from a panel of EBV-immune donors.

The location of CTL epitopes within the seven latent antigens, for which vaccinia constructs were available, was unevenly distributed. In particular, EBNA 3A and EBNA 3C were common sites for CTL recognition (11/14 donors had CTL reactivity to either of these antigens), while no epitopes were localized in EBNA 1 and EBNA 3B. Since, in the present study, Vacc.EBNA 3B was not available to test the reactivity of CTL clones from nine donors, it is not possible to draw any conclusions about the occurrence of epitopes in this protein. The immunodominance of the EBNA 3 family of proteins as a source of EBV CTL epitopes seen in this study using EBV-specific CTL clones has been confirmed in a similar study using polyclonal EBV-specific T cells (19). In all, 20 distinct CTL specificities restricted through eight different class I alleles have been defined. Interestingly, HLA A2, B7, B8, B40, and B51 alleles were each shown to present two distinct CTL epitopes derived

from different latent antigens. The ability of single MHC alleles to present multiple epitopes has also been reported with HIV (26).

Surprisingly, the specificity of a large number of EBV-specific CTL clones (68%) using the available panel of vaccinia constructs could not be defined. A possible explanation for this result is that the undefined epitopes are located within EBV latent antigens other than those encoded by the vaccinia constructs available for this study; for example, terminal proteins (TP 1 and TP 2), LMP 2, or as yet unidentified latent antigens. Alternatively, antigens associated with the EBV replicative cycle could also include CTL epitopes. These antigens are detected in only a small proportion of latently infected cells by conventional techniques. However, the exquisite sensitivity by which CTLs recognize peptide fragments (27) from viral antigen raises the possibility that low levels of replicative antigens could be processed and presented as target epitopes on LCLs. It is relevant to point out that in another related study, EBNA 3B and LMP 2 were identified as targets for EBV-specific recognition (19). These observations suggest that all latent antigens except EBNA 1 are targets for EBV-specific CTL recognition, although a disproportionate number of CTL epitopes are located within the EBNA 3 proteins.

There are two important implications for the overall biology of EBV if subsequent studies confirm that EBNA 1 does not include CTL epitopes. First, the ability of BL cells (which express only EBNA 1) to proliferate in vivo, in spite of a typically normal EBV-specific CTL response (28), is consistent with the observation that there are no CTL epitopes within EBNA 1. In vitro studies have indicated that BL cells are much less susceptible to EBV-specific CTL lysis than LCLs from the same donor (29). Down-regulation of HLA class I alleles (30) and adhesion molecules (e.g., LFA-1 and -3, and ICAM-1) (31) have been implicated as possible mechanisms for this resistance of BL cells to lysis. However, recent observations from this laboratory using peptide epitopes from EBNA 2, 3A, and 3C have demonstrated that downregulation of latent antigen expression is the most critical factor in the nonrecognition of BL cells (Khanna et al., manuscript in preparation).

The lack of detectable CTL epitopes within EBNA 1 has a second important implication in regard to the persistence of EBV in peripheral B cells. A model for the persistence of EBV in B cells has recently been proposed. A feature of this model is the existence of a long-lived, non-replicating, EBNA 1-expressing B cell (32). The observations from the present study provide a mechanism by which these cells can maintain a nonimmunogenic phenotype by not expressing the critical latent proteins needed for CTL recognition.

Of the 20 CTL epitopes localized by recombinant vaccinia, five were defined at the peptide level. In addition to three previously published epitopes (12, 13, 23), in the present study we have defined two new epitopes, one in EBNA 2 and one in EBNA 3C. Although CTL clones specific for nine epitopes recognized both type 1 and type 2 transformants, a significant number of epitopes (7/16 epitopes for which EBV type was determined) were specific for type 1 EBV. Since we have recently shown that the majority of single amino acid substitutions within CTL epitopes result in loss of recognition (33), the common isolation of type 1-specific CTL clones is not unexpected when the degree of latent antigen sequence variation between the two types is compared (7).

The present study has important implications for any future EBV vaccine designed to control IM or EBV-associated tumors. First, CTL epitopes from a spectrum of individuals are distributed throughout most of the latent proteins. Second, >60% of the CTL epitopes are located in regions outside the EBNA/LMP 1 proteins. Both of these considerations suggest that any EBV vaccine based on CTL epitopes designed to provide widespread protection will need to include not only EBNA and LMP sequences but also other regions of the genome expressed during latent infection. However, since CTLs from the majority of donors recognized EBNA 3A and EBNA 3C as target antigens, incorporation of epitopes derived from these proteins into a vaccine may protect the majority of susceptible individuals from IM. The inability of human CTLs to recognize EBNA 1 as a target antigen, often the only latent antigen expressed in BL and NPC, suggests that CTL control of these tumors will not be feasible unless the downregulation of latent antigens can be reversed. The localization of CTL epitopes within LMP, however, raises the possibility of controlling EBV-associated tumors with normal LMP expression (Hodgkin's lymphoma and some NPC) by boosting the CTL response to this antigen.

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HUMAN CYTOTOXIC T LYMPHOCYTE RESPONSES TO EPSTEIN-BARR VIRUS INFECTION

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ABSTRACT

Epstein-Barr virus (EBV) provides one of the most informative systems with which to study cytotoxic T lymphocyte (CTL) responses in humans. The virus establishes a highly immunogenic growth-transforming infection of B lymphocytes, associated with the coordinate expression of six virus-coded nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, -LP) and two latent membrane proteins (LMPs 1 and 2). This elicits both primary and memory CT8$^+$ CTL responses that are markedly skewed toward HLA allele-specific epitopes drawn from the EBNA3A, 3B, 3C subset of latent proteins, with reactivities to other antigens being generally much less frequent. This heirarchy of immunodominance among the different latent proteins may at least partly reflect their differential accessibility to the HLA class I-processing pathway. Furthermore, CTLs to some of the immunodominant epitopes involve highly conserved T cell receptor (TCR) usage, a level of focusing which evidence suggests could have immunopathological consequences from cross-reactive recognition of other target structures. EBV is associated with a range of human tumors, and there is increasing interest in the possibility of targeting such malignancies using virus-specific CTLs. The dramatic reversal of EBV-driven lymphoproliferations in bone marrow transplant patients following CTL infusion demonstrates the potential of this approach, and here we discuss prospects for its extension to other EBV-positive tumors in which the immunodominant EBNA3A, 3B, 3C proteins are not expressed.

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INTRODUCTION

Epstein-Barr virus (EBV), a human gamma herpesvirus with a marked tropism for B lymphocytes, has potent cell growth–transforming ability yet is carried by the vast majority of individuals as a life-long asymptomatic infection. The growth-transforming function of the virus maps to a specific subset of genes, the latent genes, which are coordinately expressed in the latently infected lymphoblastoid cell lines (LCLs) that arise following viral infection of resting B cells in vitro. These genes encode eight latent proteins, the nuclear antigens EBNAs 1, 2, 3A, 3B, 3C, and -LP (sometimes referred to as EBNAs 1, 2, 3, 4, 6, and 5, respectively) and the latent membrane proteins LMPs 1 and 2. Allelic polymorphisms in the EBNA2, 3A, 3B, and 3C genes define two broad types of EBV isolate, types 1 and 2 (sometimes referred to as A and B), which show subtle differences in transforming function. The role of the latent proteins in the establishment/maintenance of the transformed state has recently been reviewed (1), and here we concentrate only on their importance as targets for immune recognition.

Work on the immunology of EBV infection has for many years gone hand in hand with virological studies examining the biology of the virus-host interaction. This broader scenario is also the subject of a recent review (2) from which the following essential points can be drawn. In most human populations, primary EBV infection occurs during the first few years of life, when it is almost always asymptomatic. The tendency toward delayed primary infection now being seen in Western societies, therefore, constitutes a novel situation in evolutionary terms and, very interestingly, a proportion of these late infections are clinically manifest as infectious mononucleosis (IM). During primary infection, orally transmitted virus replicates locally in oropharyngeal epithelium, with expression of the full array of replicative (lytic cycle) genes (3), and the virus also colonizes the lymphoid system through virus-driven expansion of infected B cell clones selectively expressing the latent cycle genes (4). This results in a life-long virus carrier state in which latently infected B cells constitute the reservoir upon which viral persistence depends; periodic reactivation of B cells from latency into lytic cycle can then reinitiate replicative foci, leading to low-level shedding of infectious virus from mucosal surfaces throughout life (2).

Host immune responses are thought to be of central importance both in limiting the primary infection and in controlling the lifelong virus carrier state. Serological studies have shown that the primary and persistent phases of infection are associated with different combinations of antibody reactivities to lytic and latent antigens (2). Of these, antibodies to the major virus envelope glycoprotein gp340 warrant particular attention because of their capacity both to neutralize viral infectivity (5, 6) and to mediate antibody-dependent cellular

cytotoxicity against cells in the late phase of virus replication (7). The balance of evidence suggests that such responses do not play a major role in controlling an already established EBV infection, although they may be important in rendering such an individual immune to subsequent infection with additional exogenously transmitted virus strains. Even here, however, it is questionable whether humoral immunity alone is sufficient to confer protection (8). By contrast, the frequency with which EBV-positive lymphoproliferative disease (9) and clinically apparent virus replicative lesions (10) are seen in T cell–immunocompromised patients strongly suggests an important role for cell-mediated immune responses in the control of EBV. A number of effector mechanisms are likely to be involved in this context, and some appreciation of their diversity comes from the variety of reports in which other cell types within blood mononuclear cell preparations can either delay or prevent the virus-induced in vitro transformation of resting B cells (2). Several of these effects are seen with nonimmune as well as with virus-immune donors, and they appear to be mediated by cytokine release from nonspecifically activated T or NK-like cells. The most obvious disturbance of in vitro transformation, however, is the regression of B cell outgrowth mediated by virus-specific cytotoxic T lymphocytes (CTLs), a phenomenon seen only in cultures from virus-immune individuals and one that provides the first evidence of long-term CTL surveillance over EBV infection (11). It is this type of immunity that has received most attention in recent years and that is the subject of this review.

MEMORY CTL RESPONSES

Antigen Choice

Memory CTLs in the blood of healthy virus carriers can be reactivated in vitro by stimulation with the autologous LCL and expanded as polyclonal T cell lines or as CTL clones in interleukin 2 (IL2)–conditioned medium (12–14). The bulk of the effectors thus produced are CD8$^+$ T cells that recognize the autologous LCL but not autologous mitogen-activated B lymphoblasts and that display HLA class I restriction in their pattern of reactivity against allogeneic LCLs. The response also contains a much smaller CD4$^+$ HLA class II–restricted cytotoxic component, apparent under certain conditions of in vitro reactivation (15), but these effectors are much less well characterized. When the antigenic specificity of the dominant CD8$^+$ CTLs was assayed on targets expressing individual EBV latent proteins from recombinant vaccinia vectors (16), even the earliest studies showed a marked skewing of responses toward the EBNA3A, 3B, 3C subset of antigens (17–19). Since then the analysis has been extended to more than 60 healthy virus carriers representing a range of different HLA

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types, and in the great majority of cases, the dominant CTL reactivities are again directed toward one or more of the EBNA3A, 3B, or 3C proteins. In some cases, subdominant responses recognizing other latent proteins have been detected; the most frequent target is LMP2, less frequently EBNA2, EBNA-LP, or LMP1, and very rarely if ever EBNA1 (17–20, and references below).

These apparent differences in the immunogenicity of EBV latent proteins are interesting, but it should be stressed that studies to date have concentrated largely on Caucasian donors and on CTL responses to LCLs transformed with type 1 virus, the prevalent virus type in Western societies. Furthermore it is possible that the overall pattern of results may be disproportionately influenced by certain HLA alleles such as A3, A11, B7, B8, and B44, which are relatively common in Caucasian populations, which tend to act as strong restriction elements in the EBV system, and which present target antigens from the EBNA3A, 3B, 3C subset (18, 21–26). Interestingly, another common HLA allele, A.1, has never been observed to mediate a response in this viral system, while the most frequent allele, A2.01, tends to be a weak restriction element inducing minor reactivities against epitopes from EBNA3C or LMP2 (27–29). Recent evidence in fact suggests that HLA-B alleles may generally be more efficient than HLA-A alleles in their loading and transport of viral peptides (R Khanna, DJ Moss, unpublished data).

Antigen Processing

The concept of a hierarchy of immunodominance among the EBV latent antigens is therefore a provisional one and needs to be tested further in other human populations with a different range of HLA alleles and a different spectrum of resident virus strains. Nevertheless, one of the most striking findings to emerge from the above work, namely the extreme rarity of CD8$^+$ CTL responses to EBNA1, has been further pursued in a recent study which suggests that an internal glycine-alanine repeat (GAr) domain protects the EBNA1 protein from the HLA–class I pathway of antigen presentation (30). Thus, inserting a known CTL epitope into the EBNA1 primary sequence renders it not presentable to CD8$^+$ CTLs from the endogenously expressed chimeric protein unless the GAr domain is deleted; conversely, insertion of the GAr domain into the EBNA3B sequence greatly reduces the efficiency with which EBNA3B epitopes are presented for CTL recognition. Significantly, all natural EBV isolates do show conservation of the EBNA1 GAr domain even though this domain is not required for the principal function of EBNA1, namely maintenance of the episomal viral genome in latently infected cells (31, 32). This may reflect the importance to the virus of rendering nonimmunogenic the one viral protein whose expression is likely to be essential for virus persistence.

The implication from the above work is that accessibility to the HLA class I–processing pathway could be one important factor determining the relative

immunogenicity of antigens for CTL responses. The conventional route of HLA class I processing involves the cleavage of cytosolically expressed protein, probably via the proteosome system, the delivery of derived peptides from the cytosol into the endoplasmic reticulum via the TAP1/TAP2 transporter complex, and the subsequent binding of high affinity peptides by nascent HLA molecules to form stable peptide/HLA complexes for presentation at the cell membrane (33, 34). The cis-acting protection from processing mediated by the GAr domain of EBNA1 would most easily be explained if it affected the protein's accessibility to the initial proteolytic cleavage, presumably by the proteosome. There is no reason to suspect that EBNA1 would be similarly protected from the HLA class II processing pathway; indeed CD4+ HLA class II–restricted CTLs have been detected that map to an EBNA1 epitope and that recognize LCL cells to which EBNA1 has been supplied as an exogenous antigen (35; R Khanna, DJ Moss, unpublished data). More surprisingly, however, rare CD8+ HLA class I–restricted CTL responses to EBNA1 have now been detected in the memory of two virus-immune donors (N Blake, SP Lee, AB Rickinson, unpublished data), suggesting that the GAr-mediated protection from processing may on occasion be overridden.

A second unusual feature of antigen processing in the EBV system involves the subdominant target antigen LMP2. Thus, experiments in a TAP-negative LCL background show that, while EBNA3A, 3B, and 3C proteins are not presented for CTL recognition in this environment, some CTL epitopes within LMP2 are still presented (36, 37). This is only the second documented example of TAP-independent processing of an endogenously expressed viral protein; the first was HIV-env, a molecule that naturally translocates into the endoplasmic reticulum (ER), where it is thought to become exposed to ER proteases (38, 39). LMP2, by contrast, has short cytosolically located N- and C-termini and a central hydrophobic region composed of 12 tandemly arranged transmembrane domains with very little projection into the endoplasmic reticulum (1, 40, 41). The mechanism whereby epitopes situated within transmembrane domains can be presented in a TAP-independent manner remains to be determined. However, by analogy with topologically similar ER proteins (42–44), a fraction of newly synthesized LMP2 molecules may be subject to ubiquitin-mediated proteosomal cleavage after membrane insertion, and this may release fragments that can then access the ER lumen.

Epitope Choice

Many of the above EBV latent antigen–specific CTL responses have now been mapped to defined epitopes within the primary sequence of these proteins. A complete list of these epitopes, including several recently identified in the context of less common HLA alleles, is shown in Table 1. This serves to illustrate the concentration of HLA class I epitopes in the EBNA3A, 3B, 3C proteins

Table 1   EBV-encoded CTL Epitopes

EBV antigen	Epitope co-ordinates	Epitope sequence	HLA restriction Class I	Class II	EBV type specificity	References
Latent cycle antigens						
EBNA1	407–417	HFVGEADYFEY	B35.01		nt	unp.
	515–527	TSLYNLRRGTALA		DR1	Type 1 & 2	35
EBNA2	42–51	DTPLIPLTIF	?A2/B51		Type 1	46
	280–290	TVFYNIPPMPL		DQ2	Type 1	unp.
EBNA3A	158–166	QAKWRLQTL	B8		Type 1	25
	176–184	AYSSWMYSY	A30.02		Type 1 & 2	70
	246–253	RYSIFFDY	A24		Type 1	25
	325–333	PLRGRAYGL	B8		Type 1	24
	379–387	RPPIFIRRL	B7		Type 1	23
	406–414	LEKARGSTY	B62		Type 1	unp.
	450–458	HLAAQGMAY	?		Type 1	25
	458–466	YPLHEQHGM	B35.01		Type 1	25
	491–499	VFSDGRVAC	A29		Type 1	unp.
	502–510	VPAPAGPIV	B7		Type 1	unp.
	596–604	SVRDRLARL	A2		Type 1 & 2	25
	603–611	RLRAEAQVK	A3		Type 1 & 2	21
EBNA3B	101–115	(NPTQAPVIQLVHAVY)	A11		Type 1	22
	149–157	HRCQAIRKK	B27.05		nt	unp.
	217–225	TYSAGIVQI	A24.02		Type 1	unp.
	244–254	RRARSLSAERY	B27.02		Type 1	unp.
	399–408	AVFDRKSDAK	A11		Type 1	22
	416–424	IVTDFSVIK	A11		Type 1	22
	481–495	(LPGPQVIAVLLHEES)	A11		Type 1	22
	488–496	AVLLHEESM	B35.01		Type 1	unp.
	551–563	(DEPASTEPVHDQLL)	A11		Type 1	22
	657–666	VEITPYKPTW	B44		Type 1	unp.
	831–839	GQGGSPTAM	B62		Type 1	unp.
EBNA3C	163–171	EGGVGWRHW	B44.03		Type 1 & 2	120
	213–222	QNGALAINTF	B62		Type 2	28
	249–258	LRGKWQRRYR	B27.05		Type 1	45
	258–266	RRIYDLIEL	B27.02/.04/.05		Type 1	45
	271–278	HHIWQNLL	B39		Type 1 & 2	unp.
	281–290	EENLLDFVRF	B44.02		Type 1 & 2	26
	284–293	LLDFVRFMGV	A2.01		Type 1 & 2	28
	335–343	KEHVIQNAF	B44.02		Type 1	18
	343–351	FRKAQIQGL	B27.05		Type 1	unp.
	881–889	QPRAPIRPI	B7		Type 1 & 2	23
EBNA-LP		occasional responses identified, no epitopes defined				18
LMP1		occasional responses identified, no epitopes defined				18
LMP2	131–139	PYLFWLAAI	A23		Type 1 & 2	37
	200–208	IEDPPFNSL	B60		Type 1 & 2	29
	236–244	RRRWRRLTV	B27.04		Type 1 & 2	45
	329–337	LLWTLVVLL	A2.01		Type 1 & 2	29
	340–350	SSCSSCPLSKI	A11		Type 1 & 2	29
	419–427	TYGPVFMCL	A24		Type 1 & 2	29
	426–434	CLGGLLTMV	A2.01		Type 1 & 2	27
	442–451	VMSNTLLSAW	A25		Type 1 & 2	unp.
	453–461	LTAGFLIFL	A2.06		Type 1 & 2	29

(Continued)

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Table 1 *(Continued)*

EBV antigen	Epitope co-ordinates	Epitope sequence	HLA restriction Class I	HLA restriction Class II	EBV type specificity	References
Lytic cycle antigens						
BZLF1	190–197	RAKFKQLL	B8		nt	74
	186–201	(RKCCRAKFKQLLQHYR)	C6		nt	74
BMLF1	265–273	KDTWLDARM	?		nt	73
	280–288	GLCTLVAML	A2.01		nt	73
	397–405	DEVEFLGHY	B18		nt	73
BMRF1	86–100	(FRNLAYGRTCVLGKE)	C3		nt	73
	268–276	YRSGIIAVV	C6		nt	73
BHRF1	171–189	AGLTLSLLVICSYLFISRG		DR2	Type 1	72

Note that, after allowing for internal repeats, the number of unique amino acid residues in each of the latent cycle antigens is 372 for EBNA1, 446 for EBNA2, 944 for EBNA3A, 938 for EBNA3B, 992 for EBNA3C, 109 for EBNA-LP, 359 for LMP1 and 497 for LMP2 (calculated from the sequence of the reference type 1 EBV strain, B95.8).
[ ] Bracketed sequences indicate that the minimal epitope is not yet defined.
nt—type specificity not tested but epitope identified using CTLs from type 1 EBV-infected donors.
unp.—unpublished data from authors' laboratories.

(vis-à-vis nonpreferred antigens such as EBNA1, EBNA2, EBNA-LP, and LMP1) and also draws attention to the increasing number of subdominant responses that map to epitopes in LMP2. Such a distribution cannot be wholly ascribed to differences in size between the eight coexpressed latent proteins (see Table 1 footnote), nor to any obvious differences in their levels of expression in latently infected cells (1).

It is clear that epitope choice is highly allele-specific. Thus, although many different HLA alleles direct responses toward the EBNA3A, 3B, 3C proteins, each allele presents a particular set of CTL epitopes. The only clear example of epitope sharing involves three closely related subtypes of the B27 allele (B27.02, .04, and .05), all of which present the same immunodominant epitope from EBNA3C (45). Even here, however, each subtype also presents a second epitope that is subtype-specific (J Brooks, AB Rickinson, unpublished data). Secondly, epitope choice is highly focused in that individual alleles rarely present more than two or three peptide epitopes, one of which tends to be immunodominant. Thus a strong HLA-restricting allele such as B8 induces a response to one particular immunodominant epitope (EBNA3A 325–333) and to one subdominant epitope (EBNA3A 158–166) in almost all B8-positive individuals (24, 25, 46; DJ Moss, unpublished data); likewise A11 induces a response to EBNA3B 416–424 as the immunodominant epitope and to EBNA3B 399–408 as the subdominant epitope in most A11-positive individuals (22).

Recent studies suggest that the immunodominance of one epitope over another restricted through the same allele reflects differences in their levels of representation as peptide/HLA complexes at the LCL cell surface (47). Thus, the

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EBNA3B 416–424 peptide was at least tenfold more abundant than EBNA3B
399–408 in extracts of A11-positive LCLs. Interestingly, this appeared to reflect
the longer half-life of 416–424/A11 complexes when measured at the surface
of living cells, even though the two types of complex were equally stable under
a number of conditions in cell-free assays. This difference in representation
at the membrane could also help to explain why CTLs to the 416–424 epi-
tope kill naturally infected LCL targets much more efficiently than do CTLs
to the 399–408 epitope, whereas these two types of effector cell can recognize
peptide-loaded targets down to equally low concentrations of exogenous peptide
(47). More generally, a number of in vitro–reactivated CTL clones that map to
EBV epitopes in peptide sensitization assays and that can recognize target cells
overexpressing the relevant EBV antigen from a vaccinia vector nevertheless
do not kill the naturally infected LCL. This may again reflect the fact that, for
such clones, the level of representation of the relevant peptide/HLA complex
at the LCL surface is insufficient to trigger lysis (21).

Table 1 also indicates whether these CTL reactivities are type-specific or
cross-reactive between type 1 and type 2 virus strains. Not unexpectedly, since
the allelic forms of EBNA3A, 3B, and 3C differ at 16%, 20%, and 28% of their
residues, respectively (48), many of the immunodominant CTL responses in
type 1–infected individuals map to epitopes that are not conserved in type 2
viruses. Furthermore, the first studies to be conducted on type 2 virus–infected
individuals have likewise detected responses to epitopes in EBNA3A and in
EBNA3C that are type 2–specific (28; IS Misko, unpublished data). By com-
parison, relatively little sequence diversity is found in the LMP2 protein, and
many of the CTLs to LMP2 epitopes appear to be cross-reactive between virus
types (27, 29). As to variation among EBV strains of the same type, certain
epitopes were found to be well conserved across a range of type 1 isolates, for
example, those restricted through the B8 and B44 alleles (49, 50). Likewise the
A11 epitopes EBNA3B 416–424 and 399–408 were conserved in most type 1
strains from Caucasian and African populations; interestingly, however, these
sequences were altered in isolates from China and coastal Papua New Guinea
(51, 52), two areas where the prevalence of the A11 allele in indigenous popula-
tions is extremely high. That the changes specifically involved anchor residues
affecting peptide/A11 binding and resulted in a failure of CTL recognition
(51, 52) strongly suggested that such A11 epitope-loss mutations had been se-
lected by immune pressure in these particular communities. This remains an
intriguing possibility, but more recent data suggest that Southeast Asian virus
strains, though predominantly type 1, are clearly distinguishable from type 1
Caucasian/African isolates by means of sequence microheterogeneity at various
sites in the EBNA3A, 3B, 3C genes (50, 53; R Khanna, DJ Moss, unpublished

data). Some of these lie outside known CTL epitope regions, whereas others alter the antigenicity of epitopes restricted through HLA-alleles that are not even represented in these Southeast Asian populations. Conversely, EBV isolates from isolated highland regions of Papua New Guinea, where the A11 allele frequency is very low, show the same spectrum of A11 epitope-loss mutations as do viruses from the above highly A11-positive populations (50). It may therefore be that these differences in A11 epitope sequences are coincidental markers of a particular geographically restricted viral genotype (2) rather than the specific product of CTL-mediated selection.

T Cell Receptor Usage

Another interesting feature of EBV-induced CTL memory that has recently begun to receive attention is its T cell receptor (TCR) usage. Rearrangements of the TCR α and β chain genes during T cell development provide a vast potential for diversity in TCR $\alpha\beta$ heterodimers, particularly in the hypervariable CDR3 region that is postulated to interact directly with peptide epitopes (54). It is therefore remarkable that in several B8-positive individuals, the B8-restricted response to the immunodominant EBNA3A 325–333 epitope was found to be largely composed of clones with the same highly conserved TCR $\alpha\beta$ structure (55). Such a level of conservation in TCR usage is significantly greater than has been seen within epitope-specific CTL responses in other human viral systems such as influenza or HIV (56–58). This raises the possibility that long-term antigenic challenge with EBV, a virus (unlike influenza or HIV) that is both persistent and genetically stable, may progressively select for those memory CTLs with maximal affinity/avidity for the relevant peptide/HLA complex (see next section).

It will be interesting, therefore, to determine the level of TCR focusing within memory CTL responses to other EBV epitopes. In this context, B8-restricted memory to the subdominant EBNA3A 158–166 epitope appears to be more heterogeneous in TCR usage than that described above for the immunodominant EBNA3A 325–333 response (SL Silins, DJ Moss, unpublished data). However, the situation in relation to A11-restricted memory populations appears to be reversed, in that responses to the immunodominant EBNA3B 416–424 epitope are quite heterogeneous both within and between different A11-positive individuals, whereas responses to the subdominant EBNA3B 399–408 epitope tend to use sets of highly related TCRs (59). These authors propose that epitopes such as EBNA3B 399–408, which are present in limiting amounts on the LCL membrane (47), will tend to induce more focused responses involving only those CTLs whose receptors show high affinity/avidity for the peptide/HLA complex.

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Further analysis of the B8-restricted response to the immunodominant EBNA3A 325–333 epitope showed that all memory CTL clones with the highly conserved TCR structure also mediated cross-reactive lysis of EBV-positive or EBV-negative target cells expressing the B44.02 alloantigen (60). In fact, such is the level of this EBV epitope-specific memory in B8-positive virus carriers that their alloreactive responses to B44.02 in conventional mixed lymphocyte culture are dominated by CTLs from virus-specific memory with the relevant highly conserved TCR. This graphically illustrates how a prior history of infection with an immunogenic virus such as EBV can influence an individual's level of responsiveness to an alloantigen; such mechanisms may underlie the observed clinical association between herpesvirus status and graft-vs-host disease in bone marrow transplant patients (61). The above cross-reactivity is presumably recognizing a complex of the B44.02 alloantigen and a naturally processed self-peptide; not surprisingly, therefore, T cells with this particular TCR are not detected within the EBV-induced memory CTL population in individuals with both the B8 and B44.02 alleles. Interestingly, however, such individuals do still make a response to the EBNA3A 325–333/B8 complex through a variety of different TCRs, indicating the levels of reserve strength that exist within the TCR repertoire in the response to a particular target structure (62). More recent work has shown that cross-reactivity within the EBNA3A 325–333 response in B8-positive individuals is not limited to B44.02 only, and that certain less frequent components of the response recognize either B14 or B35.01 as alloantigens. In the latter case, some evidence suggests that the alloantigen-associated self-peptide is derived from the liver-specific cytochrome p450 (SR Burrows, DJ Moss, unpublished data). This is a particularly intriguing example of molecular mimicry with potential relevance to the pathogenesis of autoimmune hepatitis, a liver disease with a suspected EBV association and a strong link to HLA-B8 (63).

PRIMARY CTL RESPONSES

The prospective study of IM patients represents potentially one of the best opportunities to monitor the primary human CTL response to a viral infection and to follow its subsequent establishment in T cell memory. Progress in this area has been relatively slow, however, because the expanded CD8$^+$ T cell population in IM blood was found to contain several cytotoxic components in ex vivo assays; in particular the presence of CTLs showing cross-reactive recognition of alloHLA molecules made it difficult to discern any HLA class I–restricted lysis of LCL targets and hence any clear indication of EBV specificity (64–67). The specificity of the response was further called into question by a report that certain Vβ subsets were consistently expanded within the T cell

population of IM patients, suggesting the involvement of a virus-coded or virus-induced superantigen as the stimulus (68; see also next section).

A number of these issues have recently been reexamined, and a clearer picture is now emerging. First, the CD8+ population in IM does show significant expansions of up to 30% of cells from particular Vβ subsets; however, the evidence suggests that these are antigen-driven and do not constitute a superantigen-like response. Thus, different Vβ subsets are affected in different patients, and very significantly, these Vβ expansions tend to show a markedly oligoclonal rather than polyclonal pattern of TCR usage, often with a single TCR structure dominant (69). Second, IM effectors have now been screened directly for EBV antigen and epitope specificity in ex vivo cytotoxicity assays using the recombinant vaccinia vector and peptide approaches. These assays have detected unequivocally an EBV-specific CD8+ CTL response in IM that again is skewed toward a limited range of epitope peptides, most of which are derived from the EBNA3A, 3B, 3C subset of latent proteins (70). However, the primary response is not simply an exaggerated version of that subsequently seen in T cell memory; thus, the relative frequencies of individual epitope specificities may alter with the shift from primary to memory, and occasionally a particular specificity may be lost altogether. Do the oligoclonal Vβ expansions seen in IM blood correspond to EBV-specific responses, and do these particular clones enter memory? Preliminary studies on B8-positive IM patients, where there was a detectable primary response to the EBNA3A 325–333 epitope, clearly show the expansion of T cells with the same highly conserved TCR rearrangement as that already identified for this epitope in the memory of healthy carriers (69, 71). In this case, therefore, clones with the relevant TCR are present within the primary response. Longer-term studies are needed to determine whether these are preferentially selected into memory at the expense of coresident clones recognizing the same epitope but via different TCR structures.

Another interesting feature of the primary response is the presence of CTLs recognizing lytic cycle as opposed to latent cycle antigens. The first such reactivity to be described mapped to an epitope in the BHRF1 protein (an early lytic cycle antigen) but was unusual in being HLA class II-restricted and mediated by a rare CD4+ CD8+ T cell (72). It is now clear, however, that CD8+ HLA class I-restricted CTLs to immediate early or early lytic cycle antigens are frequently detectable in the blood of IM patients. The best example to date is a B8-restricted response, directed against an epitope in the immediate early protein BZLF1, which in some patients may be even more abundant than the response to the immunodominant latent cycle epitopes (73). This BZLF1-specific reactivity was actually first detected in the memory of virus carriers following in vitro stimulation with a BZLF1 peptide pool (74). Identifying the full range of lytic antigen-specific reactivities in memory may well require

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new experimental approaches because in most cases the LCLs conventionally used as stimulators of memory CTL responses in vitro contain very few lytically infected cells (1). The fact that CTL responses to immediate early and some early proteins do exist in vivo suggests that antigen-presenting function is conserved in at least some cells in the early stages of the virus replicative cycle.

IMMUNOBIOLOGY OF EBV PERSISTENCE

EBV appears to have acquired a number of unique functions that enhance its ability to colonize the B cell system in the period immediately following viral transmission, to persist as a latent infection within the B cell pool in the face of CTL surveillance, and finally to reactivate from that latently infected B cell pool into lytic cycle, thereby maintaining oropharyngeal shedding by the virus-carrying host (2).

Successful infection of a new host critically depends on the ability of the virus to access the B cell system and to amplify the load of virus-infected B cells rapidly in the short period before the CTL response comes into play. Several features of the virus contribute to that end. One is the ability of the viral envelope glycoprotein gp340 to bind to the cell surface complement receptor molecule CR2 (75, 76), an interaction that mimics aspects of C3d complement binding (77) and that targets the virus specifically to the B cell lineage. Thereafter, the virus is able both to drive the proliferation of infected cells and to enhance their expression of cell survival signals such as Bcl2 through adopting an LCL-like program of full latent gene expression (4, 78). The main source of virus involved in this early colonization of the B cell system will be virions produced from local replicative lesions in the oropharynx. In this context one of the viral genes expressed during the late phase of the lytic cycle is BCRF1, a viral homologue of the cellular IL10 gene (79, 80), and at least two biological activities of this virally encoded cytokine may work to enhance successful infection of the B cell system. First, in studies in vitro, viral IL10 can promote the efficiency of virus-induced transformation through its capacity to augment early B cell proliferation (81, 82); second, though evidence conflicts on this point (83; and AD Stuart, M Mackett, personal communication), viral IL10 may serve to impede the local generation of CTL responses either to lytically or to latently infected cells (84). Very recent studies also highlight another mechanism whereby lytically infected cells may enhance the initial stages of growth transformation in adjacent B cells. This extrapolates from the interesting in vitro finding that LCL cells, if induced into lytic cycle, can under certain conditions selectively elicit a superantigen-like response in autologous T cells of the Vβ13 subset. If this were indeed to occur in vivo in the natural setting of EBV replicative lesions, it is suggested

that cytokines derived from the Vβ13 T cell infiltrate may enhance local B cell transformation events (85).

The initial expansion of latently infected growth-transformed B cells seen in IM patients is eventually brought under control, presumably by the primary virus-specific CTL response, and a rapid fall occurs in viral load (2). However, the virus is never eliminated from the B cell pool, and a key event in the establishment of virus persistence must be the reversion of some growth-transformed B cells back to a resting state (86) and the concomitant downregulation of many of the virus latent cycle antigens that constitute the major targets for CTL attack. The cellular and/or viral controls that achieve this switch are completely unknown, and the actual pattern(s) of viral protein expression thus produced can only be surmised from transcriptional studies (4, 87, 88). The pattern appears to include EBNA1, the virus genome maintenance protein (31, 32), and/or LMP2, a protein thought to be involved in stabilization of the latent state (89, 90). Of these, EBNA1 is protected from presentation to CD8$^+$ CTLs (30), while LMP2 may not be displayed efficiently for CTL detection if antigen processing functions are themselves downregulated in a resting B cell (91). What determines the virus's ability to reactivate from this latently infected reservoir into lytic cycle is again unknown, but because the cells involved are a mobile recirculating population, it is tempting to think that physiological signals, perhaps received by B cells when infiltrating mucosal surfaces, may be used as the trigger (2). The process of reactivation and entry into a program of lytic antigen expression should then render these cells once again susceptible to CTL control, this time mediated by lytic antigen-specific effectors. Such CTLs clearly are present in virus-infected individuals, and therefore the CTLs have the potential to control rates of reactivation. However, it would not be surprising to find a mechanism whereby cells in EBV lytic cycle might at some stage become protected from CTL recognition, given the examples of herpes simplex and cytomegaloviruses, both of which employ specific lytic cycle products to interfere with the HLA class I–processing pathway (92–94).

CTL CONTROL OF EBV-ASSOCIATED TUMORS

The association between EBV and an increasing range of human tumors has been reviewed in detail elsewhere (2). Here we focus on these malignancies specifically as targets for CTL recognition.

Immunoblastic Lymphoma

Immunoblastic lymphoma (sometimes called posttransplant lymphoproliferative disease) is a B cell tumor that occurs frequently in T cell immunocompromised patients (9). The realization that these tumors are composed of

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EBV-transformed LCL-like cells expressing the full spectrum of virus latent proteins (95–97) had two important implications. First, it showed that EBV can indeed be directly oncogenic in vivo; second, it strongly suggested that the outgrowth of EBV-transformed cells would be reversed by a restoration of CTL control. The latter has now been dramatically proven in clinical practice (98–100), well ahead of the laboratory reconstruction demonstrating CTL-mediated regression of LCL outgrowth in severe combined immunodeficient mice (101). Thus in bone marrow transplant patients, where the lymphomas that arise are of donor B cell origin, the adoptive transfer either of peripheral blood mononuclear cells (98) or, more importantly, of in vitro reactivated EBV-specific CTL preparations (99) from the donor can rapidly reverse tumor growth. Furthermore, adoptively transferred CTL preparations could also be used prophylactically to reduce the EBV load in the B cell system, thereby reducing the risk of lymphoma development (99, 100). An essentially similar strategy could also be applied to solid organ transplant programs except that here any emerging lymphomas would be of recipient B cell origin and would need to be targeted by in vitro reactivated recipient CTLs; where necessary, such CTL preparations could be made from the patient's lymphocytes cryopreserved before transplantation.

Burkitt's Lymphoma

Burkitt's lymphoma (BL) is an unusual childhood tumor that in its high incidence endemic form is consistently EBV-positive (2). In marked contrast to matching LCLs, those BL cell lines that retained the original tumor cell phenotype were essentially nonimmunogenic in vitro, unable to stimulate either EBV-specific or even alloreactive T cell responses (102); furthermore, such cell lines were clearly not recognized by LCL-stimulated preparations of EBV-specific CTLs (103). The latter result was not due to any inherent resistance to cytolysis because the same lines were killed by some allospecific effectors (104) and could also be sensitized to EBV-specific lysis by exogenous loading of a relevant epitope peptide (105).

Several coincident features of BL cells appear to contribute to this immunologically silent phenotype. The inability to stimulate CTL responses in vitro probably reflects the absence or very low expression of ancillary molecules on the BL cell surface, in particular ICAM1 and LFA3 (106), which mediate adhesion to T cells via the LFA1 and CD2 pathways, respectively, and B7.1 and B7.2 (107; AB Rickinson, unpublished data) which deliver costimulatory signals to T cells via CD28 ligation (108). Interestingly, none of these aspects of the BL surface phenotype is a bar to CTL recognition per se, providing the CTL is preactivated and its relevant target structure is presented at the cell surface; in such circumstances the effector-target interaction appears to be

stabilized through ancillary adhesions involving ICAM2, an alternative LFA1 ligand constitutively expressed by BL cells (105).

The inability of EBV-specific CTLs to recognize BL targets reflects at least two additional features of this tumor. One is the well-documented downregulation of the viral genome such that only a single viral protein, EBNA1, is detectably expressed in BL cells (109). The second is a more recently discovered impairment of the HLA class I antigen presentation pathway itself. Thus the vaccinia-vectored expression of an immunogenic antigen (such as one of the EBNA3 proteins) within BL cells still does not sensitize the cells to specific CTL recognition, whereas lysis is observed if antigen processing and peptide transport are bypassed through expression of the minimal epitope peptide linked to an ER signal sequence (110). The above processing defect is associated with low expression of the TAP1/TAP2 genes, such that levels of the TAP proteins in BL cell extracts are often barely detectable compared to LCLs, and also with low expression of the HLA class I genes, such that cell surface HLA class I levels are <25% of LCL values (91, 110). Antigen processing function, as well as TAP and HLA expression, can be restored in several BL cell lines either by interferon γ treatment or, more interestingly, by vector-mediated expression of the EBV latent protein LMP1 (91). LMP1 is thought to be the major effector protein through which the virus drives resting B cells into growth (1) and, as such, must be central to the whole viral strategy for efficient amplification within the B cell system (2). Its coincident ability to upregulate the antigen processing pathway can also be seen as advantageous to the virus, however, because no benefit accrues if the virally transformed cells that initially disseminate the infection were immunologically silent and therefore able to grow out and kill the host.

It is intriguing that BL, a virally associated tumor, should display the classic immune escape phenotype. However, it is worth emphasizing that EBV-negative cases of this same malignancy (arising as rare childhood tumors outside endemic areas) show the same downregulation of ancillary molecules at the cell surface and the same deficiency in antigen processing (2, 91, 110). The immunologically silent nature of BL is therefore more likely to be a reflection of the particular progenitor cell from which the tumor arises (a germinal center B cell) (111) than of the tumor's viral association per se. Likewise the form of latency displayed by EBV in BL is not necessarily the product of immune selection but may reflect the virus's normal interaction with B cells at that particular stage of differentiation.

Nasopharyngeal Carcinoma (NPC) and Hodgkin's Disease (HD)

All cases of undifferentiated NPC both in high incidence (Southeast Asia) and low incidence areas are EBV genome-positive, as are at least 40% of cases of

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HD worldwide (2). Given that EBV-specific memory CTL responses can be
reactivated in vitro from the blood of NPC and HD patients (112, 113), this
again implies an ability of these tumors to grow in the face of CTL surveil-
lance. Immunohistochemical analysis of fresh biopsies or of archival tissue in
fact indicates that in both types of tumor the malignant cells express many of
the adhesion and costimulatory molecules necessary for effective interactions
with the T cell system and also display a TAP-positive, surface HLA class
I-positive phenotype (114–117). This suggests that antigen processing func-
tions are intact. Furthermore, recent studies with HD cell lines (albeit derived
from EBV-negative cases of the disease) have also confirmed their ability to
present vector-expressed viral antigens for CTL recognition in vitro (SP Lee,
AB Rickinson, unpublished data).

A key feature of both NPC and HD is in fact the viral phenotype. Just as
in BL, these tumors constitutively express EBNA1, but the remaining nuclear
antigens (including the immunodominant EBNA3A, 3B, 3C proteins) are down-
regulated. Unlike BL, however, LMP1 is detectable in the malignant cells in
at least a proportion of NPCs and in every EBV-positive case of HD, while the
evidence from transcription studies suggests that both types of tumor are con-
sistently LMP2-positive (2). With regard to these two virus-coded membrane
proteins, there are occasional reports of CTL memory to LMP1 in virus-immune
donors (18), but as yet the specificity of these responses has not been confirmed
at the epitope level. By contrast, an increasing number of CTL epitopes are
being defined in LMP2 for a range of HLA class I alleles such as A2.01,
A2.06, A11, A24, and B60, which are relatively common in Caucasian and/or
Southeast Asian populations (27, 29). Furthermore these epitopes are generally
conserved among type 1 and type 2 EBV isolates worldwide (27, 29), another
factor favoring their potential role as targets for a CTL-based tumor therapy.
The inability of the CTL system to reject these potentially immunogenic tumors
may be due in part to the inherent weakness of LMP2-specific responses, but
may also reflect some local cytokine-mediated suppression of CTL activation
in the vicinity of the tumor itself. An important observation in this regard was
that EBV-specific CTLs could be reactivated from the blood of patients with
EBV-positive HD but not from the tumor-infiltrating lymphocyte population
(113). The possibility needs to be kept in mind that cytokines such as cellular
IL10, known to be made by EBV-positive HD cells (118), may alter tumor
immunogenicity (84, 119). Clearly a number of issues here deserve closer
attention, particularly if in future one is to consider some form of CTL-based
therapy for such malignancies. A key objective in this latter context would be
to amplify only those components of the EBV-specific CTL response that are
directed against viral antigens actually expressed in tumor cells (120).

VACCINE STRATEGIES

The ever-increasing range of EBV-associated disease and our better understanding of virus-induced immune responses together have led to renewed interest in the development of an effective vaccine. In western societies the principal aim of such a vaccine would be protection from infectious mononucleosis. In this context, it is noteworthy that only a proportion (up to 50%) of delayed primary infections present with clinical disease. Why this should be is not known; however, a high virus load (a large dose of orally transmitted virus and/or over-expansion of the virus-transformed B cell pool beyond a critical threshold) may be a critical determinant of disease risk (2). Therefore, a vaccine achieving even a modest reduction in EBV load during primary infection may be sufficient to avert clinical symptoms. Similarly, vaccination may also reduce the immediate risk of lymphoproliferative disease in patients such as pediatric liver transplant recipients, who frequently sustain primary EBV infection from the transplanted organ or from associated blood transfusions (9). In contrast, EBV-associated tumors such as BL, NPC, and HD arise in patients years after their primary infection, and protection from these longer-term consequences would require a vaccine that ideally conferred sterile immunity and prevented the establishment of the carrier state.

Two broad approaches to EBV vaccine development are currently being considered. The first seeks to exploit the major envelope glycoprotein gp340 as the immunogen, a strategy based initially upon the observation that this glycoprotein is the principal target of the virus-neutralizing antibody response (5, 6). Various formulations of gp340 either presented as subunit antigen or expressed from recombinant viral vectors are protective against EBV-induced B cell lymphoma in the tamarin (121); the mechanism of protection in this animal model is not yet clear but appears to be due to a combination of antibody and cell-mediated responses, with recent results suggesting a role for specific CTLs (122). Encouraged by the success of these animal experiments, a first vaccine trial has been conducted in China using a live vaccinia/gp340 recombinant. Sixteen months after vaccination, all children in the control group had become EBV-infected in the normal way [detected by antibody responses to the virus capsid antigen (VCA)], whereas 6/9 vaccinated children remained anti-VCA antibody negative (123). This result, if confirmed, is important because it implies that in some vaccinees the neutralizing antibody and/or cellular responses to gp340 had indeed induced sterile immunity, at least in the short term.

An alternative approach to vaccine development is based on the induction of EBV-specific CTL immunity. Here the aim is not to prevent primary infection per se but to limit those events occurring immediately postinfection, namely virus replication in the oropharynx and/or the expansion of virus-transformed

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B cells within the lymphoid pool. As reviewed in more detail elsewhere (124),
any CTL-based vaccine strategy will likely aim toward immunization with com-
binations of defined epitopes. A first step toward this goal is to use formulations
of synthetic peptides that mimic immunodominant epitopes known to be rec-
ognized by the natural virus-induced CTL response. Indeed a human phase 1
trial is currently underway in one of our laboratories (DJ Moss) using the HLA-
B8-restricted epitope sequence FLRGRAYGL (EBNA3A 325–333) formulated
in the water-in-oil emulsion, Montanide ISA 720, and tetanus toxoid as helper
(124). This vaccine formulation is based on principles established in the murine
cytomegalovirus model, where protection from virus challenge could be demon-
strated following administration of a single immunizing dose of an immun-
odominant CTL epitope peptide (125). Obviously one of the major obstacles
of any peptide epitope approach to vaccination in humans is HLA polymorphism
because epitope choice is allele-specific. However, this obstacle might be over-
come using appropriate mixtures of synthetic epitope peptides or by construct-
ing vectors to express polypeptides in which the relevant epitope sequences are
linearly joined together. Somewhat unexpectedly, when such an EBV polyepi-
tope sequence was expressed within cells from a recombinant vaccinia vector, all
of the constituent epitopes were efficiently presented for CTL recognition (126),
indicating the potential of this approach as a vaccine strategy. More recently,
work in a murine model has shown that each of several CTL epitopes combined
in a polyepitope construct was capable of eliciting a CTL response in vivo and
could protect the animals from subsequent challenge (127). In the longer term,
it may be possible to combine the gp340-based and the CTL epitope-based
approaches to EBV vaccination by generating a chimeric protein that fuses the
important immunogenic domains of gp340 with a CTL polyepitope sequence.

FUTURE DIRECTIONS

EBV is a complex virus that can infect at least two different cell types in vivo
and, depending upon the identity of the target cell and its situation, can either
initiate lytic infection or establish one of several different forms of latency, each
involving a different program of viral antigen expression. Different arms of the
immune response are therefore likely to exercise control at different points in
the viral life cycle. As described here, recent work has concentrated on one
particular facet of host immunity, the HLA class I–restricted CTL response to
latently infected growth-transformed B cells, at the expense of other mecha-
nisms that, though less accessible experimentally, may nevertheless be equally
important. Thus HLA class II–restricted CTL responses to EBV are detectable,
but their antigenic specificities are at present poorly defined (15). Also very
little is yet known about CD4$^+$ proliferative T cell responses, apart from some

preliminary studies with individual lytic cycle antigens (128–130); yet these could play an important cytokine-mediated role in vivo. The question of CTL control over lytic cycle infection is just beginning to receive long-overdue attention, and as yet little is known about the identity of the immunodominant lytic cycle antigens or about the antigen-presenting function of lytically infected cells.

It is now two decades since the atypical lymphocytes in IM blood were identified as reactive T cells (64), yet the cellular response to primary EBV infection and its relationship to disease pathogenesis are still poorly understood. Closer examination of IM T cell populations may reveal novel virus-induced reactivities that serve either to curtail or to prolong the disease process. Some of these reactivities may well not be EBV-specific, because primary infection with other agents such as HIV or cytomegalovirus can also sometimes induce a mononucleosis-like syndrome, and the cellular response may include common immunopathological elements in each case. In rare individuals, especially young boys with the X-linked lymphoproliferative (XLP) syndrome, primary infection induces a cellular response that is ostensibly similar to that seen in classical IM, yet the disease progresses to a fatal conclusion, culminating in what appears to be a cytokine-mediated destruction of hemopoietic tissues (2, 131). The awaited cloning of the XLP gene could yet provide important clues to the pathogenesis of IM itself. Another direction for future work again relates to EBV-induced immunopathology, but this time to the possibility that damage may be mediated by the virus-specific CTL response itself. Recently discovered examples of molecular mimicry between cognate EBV peptide/HLA target structures and certain self-peptide/HLA complexes (60; SR Burrows, DJ Moss, unpublished data) not only have implications in the context of allograft rejection but also may be very important in the pathogenesis of particular autoimmune diseases (125, 132, 133).

It is likely that EBV will remain one of the principal paradigms for study of the induction and maturation of human CTL responses to viral infection, in particular persistent infection. The differential immunogenicity apparent among latent cycle antigens needs to be examined in the context of other (noncaucasian) HLA backgrounds and of other viral strains, especially type 2 strains in which the EBNA3A, 3B, 3C proteins are antigenically distinct from their type 1 counterparts. If the hierarchy of immunodominance observed to date really is a general phenomenon, what can it reveal about the biochemistry of antigen processing in LCL cells? More specifically, how complete is the protection from processing enjoyed by the EBNA1 protein, and what is the mechanism whereby a glycine-alanine copolymer domain provides such protection? If, as now seems likely, EBNA1-specific HLA class I-restricted CTLs are detectable in at least some virus-immune donors, how were they elicited? Are EBV-

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specific CTL responses actually induced by virus-transformed LCL-like cells in vivo or by a different antigen-presenting cell population?

Questioning the in vivo relevance of in vitro findings is a recurrent theme in the EBV literature, not least in the field of immunology. More than anything, this reflects the absence of a good animal model that mimics natural EBV infection and persistence more closely than do the artificial situations produced on experimental infection of certain New World primate species (121, 134). If such a model existed, one could hope to dissect the different components of immune responsiveness by cell depletion experiments and to look at the relative importance of these different components in primary versus persistent infection. In this context it is very interesting that many species of Old World primate actually carry their own B lymphotropic herpesviruses that are closely related to EBV in terms of genome structure (1), that have B cell growth transforming ability, and that are likewise kept in check by immune T cell surveillance in vivo (135). Not only does their existence have important implications for our understanding of the evolution of EBV, it also opens up the possibility of establishing tractable models that would recapitulate all the essential features of EBV infection in humans.

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Quantification of antigen specific CD8+ T cells using an ELISPOT assay

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Abstract

An ELISPOT assay to detect and determine the number of antigen specific CD8+ T cells was standardized using cloned murine CD8+ T cells specific for the epitope SYVPSAEQI of a rodent malaria antigen. This assay is based on the detection of IFN-γ secretion by single cells after their stimulation with antigen. The interferon secretion is visualized as spots revealed by using enzyme labeled anti-IFN-γ monoclonal antibodies. Using known numbers of cloned murine CD8+ T cells it was determined that the assay detects 80–95% of these CD8+ T cells. The optimal culture conditions for the stimulation of the CD8+ T cells were determined and the antigen concentration, number of antigen presenting cells and supplement of growth factors required to perform the assay were defined. This ELISPOT assay can be performed with spleen cells from immunized mice, and provide the precise number of antigen specific CD8+ T cells present in mixed lymphocyte populations. This method is more sensitive than the chromium-51 release assay, and much simpler than the conventional precursor frequency analysis, providing the number of antigen specific CD8+ T cells in 36–48 h.

Keywords: CD8+ T cell; ELISPOT; IFN-γ; Synthetic peptide; Recombinant vaccinia virus; Malaria

1. Introduction

CD8+ T lymphocytes mediate one of the most important effector mechanisms of immunity against intracellular pathogens such as viruses, bacteria and parasites. These T cells recognize small peptides, derived from microbial antigens, which are presented on the surface of infected cells by class I MHC molecules.

Investigations on the mode of induction, and persistence of antigen specific CD8+ T cells, have been severely hampered by the lack of a simple assay capable of providing reliable quantitative data on these T cells. A widely used method for the detection and measurement of cytotoxic

Abbreviations: CS, circumsporozoite; PYCS, recombinant vaccinia virus; IFN-γ, interferon-γ; MHC, major histocompatibility complex; DMEM, Dulbecco's modified eagle medium; PBS/T, phosphate-buffered saline containing 0.05% Tween 20; APC, antigen presenting cell.

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CD8+ T cell activity is the ^{51}Cr release assay (Grabstein and Chen, 1980). This assay provides semi-quantitative data and is most useful for the detection of cytotoxic T cells. However, the ^{51}Cr release assay is inadequate for studies which require the determination of the number of antigen-specific CD8+ T cells in a mixed lymphoid cell population. The best assay currently in use for such quantitative studies is the precursor frequency analysis, using the limiting dilution technique (Taswell, 1981). While it is generally accepted that this is an excellent method, its use is severely limited by the fact that it is very time consuming and laborious, and therefore unsuitable for studies which require the simultaneous comparison of several sets of experimental data.

We report here the standardization of an alternative method to determine the number of antigen specific CD8+ T cells. This method was adapted from the ELISPOT assay described for the detection of mitogen activated CD4+ T cells (Taguchi et al., 1990) and is based on the visualization of IFN-γ secretion by single CD8+ T cells after their in vitro stimulation with antigen. The IFN-γ secretion by individual cells is visualized as spots revealed by using enzyme labeled anti-IFN-γ monoclonal antibodies. Using cloned murine CD8+ T cells, which recognize a well defined epitope, we determined the optimal conditions for in vitro culture, antigen stimulation and detection of IFN-γ secretion by CD8+ T cells. This assay is a rapid, highly sensitive method providing a quantitative assessment of the number of activated antigen specific CD8+ cells. It differs in some important methodological aspects from the originally described ELISPOT assay used for the enumeration of IFN-γ secreting CD4+ T cells (Taguchi et al., 1990; Xu-Amano et al., 1992).

2. Material and methods

2.1. Cells and cell culture medium

The CD8+ T cell clone YA26, derived from a BALB/c (H-2^d) mouse immunized with *Plasmodium yoelii* sporozoites, was used throughout these experiments. The generation, the antigen specificity and the conditions for in vitro culture of this T cell clone have been described in detail elsewhere (Rodrigues et al., 1991,1992). Briefly, YA26 cells were cultured in DMEM high glucose (Gibco) medium, supplemented with 10% fetal calf serum (FCS), 10 mM Hepes, 2% EL-4 cell supernatant, 10^{-5} M 2-mercaptoethanol (2-ME) and 2 mM L-glutamine. P815 cells (H-2^d) were used as target cells for both the ^{51}Cr release and the ELISPOT assays. The EL-4 supernatant used as source of interleukins was obtained by stimulating EL-4 cells with PMA. It was used at a final concentration of 2% which contains 30 U of IL-2/ml.

2.2. ELISPOT assay for the detection of IFN-γ producing YA26 cells

96-well nitrocellulose plates (Mililiter HA, Millipore, Bedford, MA) were coated with 10 μg/ml of the anti-mouse IFN-γ mAb R4 (from the American Type Culture Collection, Bethesda, MD), in 75 μl of phosphate-buffered saline (PBS). After overnight incubation at room temperature, the wells were repeatedly washed with culture medium and 100 μl of DMEM high glucose, medium containing 10% FCS and 10 mM Hepes, were added to each well for 1 h at 37°C. Known numbers of CD8+ T cells of the YA26 clone were then suspended in culture medium and placed into the antibody-coated wells.

P815 cells were incubated with the synthetic peptide SYVPSAEQI, for 1 h at 37°C. After repeated washings with culture medium, these peptide-pulsed P815 cells were irradiated, and added to the ELISPOT wells. P815 cells, not pulsed with peptide, were used as controls of antigen independent IFN-γ secretion.

After incubation at 37°C and 5% CO$_2$ for 24–28 h, these plates were extensively washed with PBS containing 0.05% of Tween 20 (PBS/T). The wells were then incubated with a solution of 5 μg/ml of biotinylated anti-mouse IFN-γ mAb XMG1.2 (Pharmingen, CA) in PBS/T. The plates were incubated overnight at 4°C, washed with PBS/T and then 100 μl of peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories, KPL, Gaithesburg, MD), at a 1/800 dilution in

PBS/T, were added to each well. After 1 h incubation the wells were washed twice with PBS/T and twice with PBS. The spots were developed by adding 50 mM Tris-HCl at pH 7.5, containing 1 mg/ml of the substrate 3,3'-di-aminobenzidine-tetra-hydrochloride dihydrate (DAB), and 5 μl/10 ml of 30% H_2O_2 to each well. After 10–15 min the number of spots were determined with the aid of a stereomicroscope.

2.3. Chromium release assay

The chromium release assay was performed as described elsewhere (Rodrigues et al., 1991). Briefly, 10^6 P815 cells were labeled with 250 μCi ^{51}Cr in FCS, for 1 h at 37°C. YA26 cells were incubated, at different ratios, with 2.5×10^3 ^{51}Cr labeled P815 cells per well, in the presence or absence of 10^{-7} M peptide SYVPSAEQI. After 5 h of incubation at 37°C, the supernatants were collected with the aid of a semi-automatic harvester (Skatron, Sterling, VA), and the number of counts determined. The percentage of specific lysis was calculated as previously described (Rodrigues et al., 1991).

2.4. Immunization of mice with a recombinant vaccinia virus expressing the SYVPSAEQI epitope and determination of splenic antigen specific CD8+ T cells by the ELISPOT assay

In experiments designed to determine the number of CD8+ epitope-specific IFN-γ secreting T cells, groups of three BALB/c mice were immunized i.p. with 5×10^7 pfu of recombinant vaccinia virus expressing the *P. yoelii* CS protein (PYCS) (Li et al., 1993). 11 days after the immunization, the mice were killed, their spleens removed, and the number of epitope specific IFN-γ producing cells determined. We used freshly isolated spleen cells and also further in vitro stimulated spleen cells. Different concentrations of freshly isolated splenocytes, starting at 5×10^5 cells/well, were screened for antigen specific CD8+ cells using the standardized ELISPOT assay. In addition, 5×10^7 spleen cells of the same pool were expanded in vitro for 6 days with 3×10^6 P815 cells pulsed with 10^{-7} M peptide SYVPSAEQI. After this expansion, a series of different concentrations of these splenocytes,

starting with 1.25×10^5 cells/well, were analyzed by the standardized ELISPOT assay.

2.5. Precursor frequency analysis

BALB/c mice were immunized with 5×10^7 pfu of recombinant vaccinia PYCS, as described for previous experiments. 30 days after immunization, spleen cells were used to perform the ELISPOT assay and the precursor frequency analysis. The ELISPOT was performed following the procedures described for the previous experiments. The precursor frequency analysis was performed as previously described (Ceredig et al., 1987). Briefly, 0, 150, 300, 600, 1200 and 2400 spleen cells from immunized mice were cultured with 7×10^5 irradiated syngeneic normal spleen cells and 2×10^5 irradiated peptide-pulsed P815 target cells, in 96-well flat-bottom culture plates. As culture medium, we used DMEM supplemented with 10% FCS and 2.5% EL-4 supernatant. At day 8, the contents from each well were split into 96-well round bottom plates to test the cytotoxic activity against peptide-pulsed ^{51}Cr labeled P815 target cells. As control, we used radiolabeled target cells not pulsed with peptide. After 6 h the ^{51}Cr release was measured and cultures were considered positive if the level of specific ^{51}Cr release was greater, by three standard deviations, than the release detected from radiolabeled target cells incubated without spleen cells. The frequency of antigen specific CD8+ T cells was calculated as previously described (Quintans and Lefkovits, 1973).

3. Results

3.1. Sensitivity of detection of cloned CD8+ T cells by the ELISPOT and by the ^{51}Cr release assay

A murine CD8+ T cell clone, YA26, which recognizes a class I MHC restricted epitope (SYVPSAEQI), of the CS protein of *P. yoelii* (Rodrigues et al., 1991), was used to determine the level of sensitivity of detection of antigen specific CD8+ T cells by the ELISPOT and the ^{51}Cr assay.

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Fig. 1. *Top panel:* detection of IFN-γ secretion following antigen activation of cloned CD8⁻ T cells (YA26). Variable numbers of cells of the YA26 clone were incubated with 10⁵ P815 cells pulsed with SYVPSAEQI peptide, at a final concentration of 10⁻⁶ M. After 28 h of incubation at 37°C and 5% CO_2, the wells were washed and the presence of IFN-γ spots revealed as described in the materials and methods section. To one series of these wells a 2% EL-4 supernatant was added (●), whereas in an otherwise identical series, the EL-4 supernatant was ommitted (▽). Each point represents the mean of triplicates ± SE. *Middle panel:* antigen specificity of the IFN-γ spots. 300 YA26 cells were incubated with P815 target cells, as described for the *top panel*. The IFN-γ spots were clearly visible when the YA26 cells were incubated with target cells pulsed with the SYVPSAEQI peptide (B), while they are absent when target cells without peptide were used (A). *Bottom panel:* ⁵¹Cr release assay using YA26 T cells and P815 target cells. Variable numbers of the T cell clone YA26 were incubated for 5 h with 2.5 × 10³ radiolabelled P815 target cells, which had either not been pulsed (O) or were pulsed with peptide (●). Each point represents the mean of triplicates.

For this purpose known numbers of cloned CD8$^+$ T cells were incubated in nitrocellulose-based culture wells (ELISPOT wells), with antigen (peptide)-coated P815 target cells. These wells had earlier been coated with mAb to murine IFN-γ, as described (Taguchi et al., 1990). After 24–28 h of incubation at 37°C and 5% CO$_2$, the wells were washed, incubated with biotinylated anti IFN-γ antibody, followed by the addition of avidin-peroxidase and a peroxidase substrate.

As seen in Fig. 1 (top panel), we obtained an excellent correlation between the number of cloned CD8$^+$ T cells placed into each well and the number of IFN-γ spots. In fact, this assay detected between 80 and 95% of the cells of a given well. We found that addition of an EL-4 supernatant to the culture medium, to provide a supplement of interleukins was important to ensure optimal production of IFN-γ by the CD8$^+$ T cells. In the absence of the EL-4 supernatant, the number of spots, we detected, accounted for only 50–60% of the total number of the CD8$^+$ cells of each well.

The IFN-γ spots, detected by this assay, were clearly antigen specific, since few or no spots were observed upon incubation of the CD8$^+$ T cells with P815 target cells, in the absence of the peptide (Fig. 1 (middle panel)). Thus, the presence of antigen appears to be essential to trigger IFN-γ production, while the interleukins provided by the EL-4 supernatant, though not strictly necessary, significantly improve production of this cytokine.

Comparing the sensitivity of the ELISPOT (Fig. 1 (top panel)) with the ^{51}Cr release assay (Fig. 1 (bottom panel)), it became clear that under optimal conditions the ELISPOT can detect small numbers of activated CD8$^+$ T cells, namely as little as 20–30 antigen-specific CD8$^+$ lymphocytes per well. In contrast, 10^3 or more of these CD8$^+$ cells were required to produce a clear signal in the ^{51}Cr release assay. Most importantly, the ELISPOT allows the determination of the number of antigen-specific cells, which can not be done by the use of the chromium-release assay.

3.2. Optimal conditions for the antigen stimulation of CD8$^+$ cells in the ELISPOT assay

There are significant methodological differences between the earlier described ELISPOT assay for the detection of IFN-γ secreting T cells (Taguchi et al., 1990; Xu-Amano et al., 1992; Di Fabio et al., 1994) and the ELISPOT we standardized for the detection of CD8$^+$ T cells. In the previously described ELISPOT assays, the T cells are first stimulated with mitogen or antigen in standard culture plates, during 1 day or more, before being placed in the ELISPOT wells. In contrast, we found that antigen-specific IFN-γ secreting CD8$^+$ T cells can only be detected if they are placed in the ELISPOT wells, immediately after being mixed with antigen-coated target cells, i.e., without pre-incubation in a standard culture plate. As shown in Fig. 2, if CD8$^+$ T cells are stimulated with antigen 1 or 7 days before being placed in the ELISPOT wells, the γ-interferon spots can not be detected, unless these cells are mixed with antigen coated target cells just before being placed in the ELISPOT wells.

The number of detectable spots produced by antigen specific CD8$^+$ T cells is strictly dependent on the concentration of antigen used to

Fig. 2. Number of antigen-specific IFN-γ producing CD8$^+$ T cells detected antigenic stimulation in vitro for either for 1 or 7 days. YA26 CD8$^+$ T cells were stimulated with antigen for 1 day and then transferred to ELISPOT wells, immediately after mixing them with P815 target cells pulsed with (♦) or without peptide (▼). In another series, YA26 CD8$^-$ T cells were stimulated with antigen for 7 days and then placed into ELISPOT wells immediately after adding P815 cells pulsed with (▽) or without SYVPSAEQI peptide (□). Each point represents the mean of triplicates ± SE.

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Fig. 3. Optimal concentrations of peptide SYVPSAEQI for the detection of CD8$^+$ YA26 cells by the ELISPOT assay. Variable numbers of YA26 cells per well were incubated with 10^5 P815 cells pulsed with different concentrations of peptide. The peptide was used at concentrations of 10^{-6} M (●), 10^{-7} M (▽), 10^{-8} M (▼), 10^{-9} M (□) and 10^{-10} M (■). Each point represents the mean of triplicates ± SE.

Fig. 4. Effect of the number of antigen-presenting cells (P815) on the number of IFN-γ producing cells detected by the CD8+ ELISPOT assay. Twenty to 130 YA26 cells were incubated with 10^6 (●), 10^5 (▽), 10^4 (▼) or 10^3 (□) P815 cells pulsed with peptide SYVPSAEQI at a concentration of 10^{-6} M. Each point represents the mean of triplicates ± SE.

pulse the P815 cells. As shown in Fig. 3, a maximal number of interferon spots can be detected when target cells are pulsed with peptide concentrations ranging from 10^{-6} to 10^{-8} M. A significant decrease in the number of IFN-γ spots, or their absence, occured when peptide concentrations of 10^{-9} or 10^{-10} M were used, respectively.

3.3. Sensitive detection and enumeration of CD8$^+$ antigen specific T cells

The number of antigen presenting cells (APC) used in the assay is also an important variable. Their optimal concentration appears to be 10^5 P815 cells per well. The use of either a larger or smaller number of target cells decreased considerably the number of IFN-γ spots (Fig. 4).

The type of antigen presenting cells used in the ELISPOT assay appears to affect only to a small degree the generation of IFN-γ spots. As seen in Fig. 5, the best APC for the activation of the Y26 CD8$^-$ T cells appears to be the P815 mastocytoma cell line. However, rather similar results were obtained when we used other types of APC such as the macrophage cell line J-774 (ATCC: TIB-67), or the B-cell lymphoma derived A20 cell line (ATCC: TIB-208).

3.4. Detection of antigen-specific CD8$^+$ T cells in the spleens of immunized mice

With a view on the use of this assay for the detection of antigen-specific CD8$^+$ T cells of immunized mice, we determined the possible influence that unfractionated spleen cells may have

Fig. 5. ELISPOT assay performed using different types of antigen presenting cells (APCs). 100 YA26 CD8$^+$ T cells were incubated with 10^5 APC of different cell types (P815, A20 or J774) expressing the H-2K^d MHC molecules. With each cell line the assay was performed in the presence and absence of peptide. Each bar represents the mean of duplicates ± SE.

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Fig. 6. The presence of naive spleen cells does not interfere with the sensitivity of the CD8⁻ ELISPOT assay. 100 YA26 cells plus 10⁵ P815 cells, pulsed with 10⁻⁶ M peptide, were incubated with various numbers of spleen cells obtained from normal BALB/c mice. Each point represents the mean of triplicates ± SE.

on this detection. A constant number of cloned CD8$^+$ T cells and peptide-coated target cells were incubated with varying numbers of naive spleen cells. As can be seen in Fig. 6, the presence of variable, even very large numbers of

spleen cells did not affect the IFN-γ secretion by CD8$^+$ T cells, in this assay.

We then used this ELISPOT to determine the number of antigen-specific CD8$^+$ T cells in the spleen of mice immunized with a recombinant vaccinia virus expressing the epitope, SYVP-SAEQI in the context of the CS protein (Li et al., 1993). Eleven days after the immunization of mice with 5×10^7 pfu of this recombinant vaccinia virus, their splenic lymphocytes were obtained and the number of epitope specific CD8$^+$ splenic T cells was determined. We used both fresh spleen cells (immediate ELISPOT) and spleen cells after in vitro stimulation with antigen (ELISPOT after expansion).

The immediate ELISPOT assay, performed with freshly obtained spleen cells, revealed the presence of epitope-specific CD8$^+$ T cells induced by the immunization with the recombinant viruses (Fig. 7 (left panel)). Most of the IFN-γ spots detected in the assay were antigen-specific, since only very few spots can be observed when these immune spleen cells were incubated with target cells in the absence of antigen. Furthermore, no antigen specific IFN-γ spots could be

Fig. 7. Detection of antigen specific CD8$^+$ T cells in the spleen of mice immunized with vaccinia virus PYCS. 11 days after immunization of BALB/c mice with 5×10^7 pfu of recombinant vaccinia virus PYCS, their spleen cells were obtained. A CD8$^+$ ELISPOT assay was performed, either on the same day (left panel, immediate ELISPOT), or after 6 days in culture of these cells (right panel, expanded ELISPOT). For the expanded ELISPOT, 5x10^7 spleen cells were cultured for 6 days in 10 ml DMEM of a high glucose medium containing EL-4 supernatant, together with 3x10^6 P815 cells pulsed with 10^{-6} M peptide. The subsequent phase of these assays, conducted in the ELISPOT wells, was performed in the presence and in the absence of EL-4 supernatant, and in the presence or absence of peptide-pulsed target cells (specificity control). Each bar represents the mean of triplicates ± SE.

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detected upon immunization of mice with the wild type vaccinia virus (not shown). Not surprisingly, the chromium release assay performed with these freshly obtained spleen cells failed to reveal the presence of antigen-specific cytotoxic activity (data not shown). As expected, a greater number of antigen specific CD8$^+$ T cells was detected when the ELISPOT was performed using the spleen cells 6 days after their in vitro stimulation with antigen (Fig. 7 (right panel)).

In agreement with earlier experiments, performed with cloned CD8$^+$ T cells, we determined that the addition of 2% EL-4 supernatant to the spleen cells, improved considerably the detection of IFN-γ secreting cells. This improvement was particularly striking when fresh spleen cells were used in the assay (Fig. 7 (left panel)). In the case of the spleen cells expanded in vitro by antigen stimulation, the situation was not as clear. As shown in Fig. 7 (right panel), the use of EL-4 supernatant increased appreciably the number of IFN-γ producing cells. However, the addition of the EL-4 supernatant also resulted in a significant increase in the number of cells which secreted IFN-γ in the absence of peptide, i.e., in a seemingly antigen-independent fashion.

Finally, we determined the number of antigen specific CD8$^+$ T cells using both, the ELISPOT assay and the standard precursor frequency analysis using the limiting dilution technique. For this purpose, mice were immunized with the recombinant vaccinia virus and 30 days later their spleen were used to perform these assays. As seen in Fig. 8, the number of antigen specific CD8$^+$ T cells detected by both assays was very similar. This result further validates the use of the ELISPOT assay for the quantification of antigen specific CD8$^+$ T cells.

4. Discussion

Here we describe the standardization of a very sensitive, quantitative ELISPOT assay for the detection and determination of the number of antigen-specific CD8$^+$ T cells. In order to perform this ELISPOT assay with the best possible results, we standardized it by using a clone (YA26) of CD8$^+$ T cells of defined epitope specificity, so that a known number of these cells were placed in the ELISPOT wells. This permitted us to de-

Fig. 8. Quantification of antigen specific CD8$^+$ T cells using the ELISPOT assay and the limiting dilution analysis. BALB/c mice were immunized with 5x10^7 pfu of recombinant vaccinia virus PYCS expressing the SYVPSAEQI epitope. Thirty days after immunization the immune spleen cells were used to determine the number of IFN-γ producing antigen specific CD8$^+$ T cells using the immediate ELISPOT assay and the standard cytotoxic T lymphocyte precursor frequency analysis (CTLp) using the limiting dilution technique. In both assays, the number of CD8$^+$ T cells was determined in the presence and absence of the peptide SYVPSAEQI.

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termine precisely the sensitivity of this method, and to establish optimal conditions for the in vitro culture and antigen stimulation of these cells.

One of the steps that appears to be crucial for the detection of CD8^{+} T cell derived IFN-γ spots is the need for mixing the CD8^{+} T cells and peptide-coated target cells just prior to placing them in the ELISPOT wells, avoiding the cell transfer and consequent loss of secreted IFN-γ. This differs from the ELISPOT used for the detection of CD4^{+} T cells, in which it is necessary to stimulate the immune T cells with antigen and APC one or more days before transferring these mixed cell population into the ELISPOT wells (Xu-Amano et al., 1992)

We identified additional variables which greatly affect the efficacy of the ELISPOT assay in detecting antigen-specific CD8^{+} cells. Our results clearly demonstrate that addition of exogenous interleukins (EL-4 cell supernatant) to the culture medium, increases greatly the sensitivity of this assay. The mechanisms by which the interleukins enhance the functional activity of these cells remains to be determined. In this regard it is noteworthy that the absence of EL-4 supernatant during the time it takes to perform the ELISPOT, does not significantly impair the viability of the CD8^{+} T cells (data not shown). The antigen concentration used to pulse the target cells, and the number of antigen presenting cells used in the assay, are also variables which influence appreciably the number of detectable IFN-γ spots.

In the context of applying the ELISPOT assay for the determination of the number of antigen specific CD8^{+} T cells, it is necessary to establish experimental conditions which ensure that CD8^{+} T cells are the only IFN-γ secreting cells being detected. In the case of clone YA26, which we used for the standardization, it is well established that the epitope SYVPSAEQI is recognized by CD8^{+} T cells in the context of H-2K[d] class I MHC molecules, and that this sequence is not recognized by CD4^{+} T cells (Romero et al., 1990; Rodrigues et al., 1994). However, when larger antigens or complex antigenic mixtures are used, it will be necessary to exclude the possibility of concomitant activation of both CD4^{+} and CD8^{+} T cells.

This problem can in part be circumvented by using antigen presenting cells, which express only class I not class II MHC molecules, such as the P815 mastocytoma cell line. As described in the methods section, these cells are extensively washed after 1 h of incubation with antigen, prior to incubation with the T cells. Under these conditions, it is expected that the antigen, introduced into the culture, is all bound to the P815 target cells.

Another alternative is to perform this assay using CD4^{+} T cell depleted lymphoid cells. These CD4^{+} depleted cell populations can be obtained by (a) in vivo treatment of the immunized mice with anti-CD4 antibodies, or (b) in vitro pre-incubation of the T cell preparations with anti-CD4 antibodies. In our system, as expected, neither the in vivo nor the in vitro depletion of CD4^{+} T cells affected the detection of antigen-specific CD8^{+} T cells. In sharp contrast, when we used CD8^{+} depleted spleen cells the opposite occurred, i.e., IFN-γ spots could no longer be detected (Rodrigues et al., 1994).

The ELISPOT assay for the detection of antigen specific CD8^{+} T cells, appears well suited for monitoring quantitatively various basic immunologic phenomena, such as the kinetics of induction of effector and of memory CD8^{+} T cells, as well as the organ compartmentalization, homing and trafficking of these cells. This methodology is expected to have broad applications in vaccine development, particularly for the evaluation of the immunogenic properties of sub-unit vaccines designed to induce CD8^{−} T cells against intracellular microbial pathogens. Recently we have used this assay to evaluate and compare the immunogenicity of various recombinant vaccinia and influenza viruses, expressing the SYVPSAEQI epitope in different structural contexts (Rodrigues et al., 1994).

In conclusion this ELISPOT assay has clear advantages over the standard chromium-release methodology since it is more sensitive, and permits the determination of the number of antigen-specific cells. It also compares favorably

with the standard frequency precursor analysis which uses the limiting dilution technique, because it is much simpler to perform, is less time consuming and less labor intensive, the results becoming available within 36–48 h.

Acknowledgements

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<div align="right">**PATENT**</div>

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Group Art Unit: 1644

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Atty. Dkt. No.: FBRC:004/TMB

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A cytotoxic T cell polyepitope based vaccine against HIV delivered by modified vaccinia anchara

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SUMMARY

There is now a considerable compelling indirect evidence that αβ CD8+ cytotoxic T lymphocytes (CTL) have an important role in preventing HIV infection and/or slowing progression to AIDS. An ideal HIV CTL based vaccine might induce CTL specific for multiple epitopes and/or multiple epitope variants and thereby generate activity against all challenge isolates and/or pre-empt potential escape mutants. The polyepitope or "polytope"approach, whereby multiple contiguous minimal CD8 CTL epitopes are codelivered in a single artificial construct, has now been repeatedly shown to be capable of inducing CTL specific for multiple epitopes. Several vectors, including modified vaccinia virus Ankara (MVA) are currently being evaluated for their potential to deliver polytope constructs in human clinical trials.

The polytope approach for multiple CTL epitope delivery. The design of minigene vaccines containing multiple CTL epitopes was initiated by Whitton et al., 1993, who combined two potentially independently initiated minigenes, which coded for sequences containing single CTL epitopes. Recently we showed that each epitope, within an artificial polyepitope (or polytope) protein comprised of multiple contiguous minimal CTL epitopes in sequence, was individually processes and presented (Thomson et al., 1996). Such polytope constructs, delivered by vaccinia or DNA, were also able to induce independent CTL responses to all the epitopes in the construct and generated CTL which were protective in several virus and tumour models (Thomson et al., 1996,1998: WO96/0314).

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The polytope approach has now been shown to work for a variety of epitopes, diseases and vectors including, HIV and Modified Vaccinia Anchora (MVA) (Hanke et al., 1998), malaria and Ty particles (Gilbert et al., 1997), different viruses and vaccinia (An and Whitton, 1997), influenza and lipopeptides (Sauzet et al., 1995) and tumours and recombinant adenovirus (Toes et al., 1996).

Are flanking sequences required. In all the polytope constructs described above, except those reported by Sauzet et al., 1995 and Toes et al. 1996, multiple minimal CTL epitopes were directly conjoined without the inclusion between the epitopes of (i) any intervening spacer amino acid sequences or (ii) sequences naturally found to flank the epitopes in their proteins of origin. The addition of universally processable linkers between individual CTL epitopes, for example three alanines (Toes et al. 1996), was considered potentially beneficial as profound influences of flanking sequences on CTL epitope processing have been reported (Bergmann et al., 1996, Suhrbier, 1997). Negative influences on the processing of specific epitopes can, however, often be ascribed to the presence of glycine or proline residues adjacent to the minimal epitope (Del Val et al., 1991, Hahn et al.,1992, Eggers et al., 1995, Bergmann et al., 1996, Yellen-Shaw et al., 1997). Proteolytic cleavage around glycine and proline residues (especially when in the P1 position) tends to be poor for many non specific proteases, including proteasomes (Niedermann et al., 1996). Their negative influence is, however, unlikely to be seen in polytope constructs, since they almost never occupy the C terminus of CTL epitopes (Rammensee et al., 1995).

Proteolytic generation of CTL epitopes principly involves the proteosome and associated proteins, LMP 2, LMP7 and PA28, which alter the cleavage specificities (Dick et al., 1996). Non proteosomal cytoplasmic proteases may also contribute to generation of immunogenic peptides (Lopez and Del Val, 1997) as may proteolytic enzymes within the ER (Elliott et al., 1995). The ability of every epitope within several polytope constructs made without spacers or linkers to be processed and presented, strongly suggest that proteolysis and transport of epitopes into the ER is governed primarily by the intrinsic qualities of the epitope rather than by the sequences flanking them (Hahn et al., 1992, Mylin, et al. 1995, Sijts, et al. 1996, Niedermann et al., 1996, Fu, et al. 1998).

Polytope vaccines against HIV/AIDS. There is now a considerable body of compelling indirect evidence that CTL have a role in preventing or limiting (or even clearing) (i) initial HIV infection and (ii) progression to AIDS (Rowland-Jones, et al. 1997, Goulder, et al. 1997). A recombinant protein based HIV vaccine covering the epitope variants within one geographical region and containing enough epitopes to cover the HLA diversity of the population, would need to contain a considerable number of recombinant antigens. In contrast, a relatively small HIV polytope vaccine would be able to co-deliver multiple epitopes and/or multiple epitope variants. In a prophylactic setting this approach might ensure CTL activity against different challenge variants, and in a therapeutic setting might pre-empt potential escape mutants (Goulder et al., 1997, Mortara , et al. 1998). We are currently testing a polytope containing multiple HIV HLA A2 epitopes (TABLE I) in HLA A2 transgenic mice to illustrate immunogenicity of each epitope prior to human clinical trials.

TABLE I. HLA A2 restricted HIV epitopes the polytope.

EPITOPE	Sequence	EPITOPE	Sequence
pol 476-484	ILKEPVHGV	*gag* 77-85	SLYNTVATL
gp120 120-128	KLTPLCVTL	*nef* 180-189	VLEWRFDSRL
pol 346-354	VIYQYMDDL	*nef* 190-198	AFHHVAREL
nef 157-166	PLTFGWCYKL		

HIV polytope delivery by modified vaccinia virus Ankara (MVA) A large number of vaccination modalities have been developed which are capable of delivering recombinant proteins for the induction of CTL, few have emerged as safe and effective inducers of CTL in humans. MVA was generated by longterm passage of the Ankara strain of vaccinia virus on chicken embryo fibroblasts (Mayr et al. 1975), contains six major genomic DNA deletions (Meyer et al., 1991) and most importantly has been administered to over 120,000 humans, including many

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patients at risk for conventional vaccination against smallpox (Mayr et al., 1978). Methods for generating recombinant MVA viruses have recently been developed whereby foreign genes are inserted at the site of a naturally occurring deletion within the MVA genome (Sutter & Moss 1992, Sutter et al., 1995, WO 97/02355). Recombinant MVA constructs have been shown to induce CTL in mice (Sutter et al., 1994, Hanke et al., 1998; Schneider et al., 1998) and are soon to enter into phase I human trials. Characteristics which qualify MVA-based vectors as gene delivery system with excellent safety profile and high immunogenicity of expressed antigens include: (i) the inherent avirulence of MVA even in immunosuppressed organisms (Werner et al., 1980), (ii) its inability to produce viral progeny in primary human cells (Drexler, et al. 1998), (iii) the high level expression of heterologous genes despite replication deficiency (Sutter, et al. 1992), (iv) the finding that MVA fails to produce soluble receptors for IFN-γ, IFN-α/β, and TNF-α (Schneider et al., 1998), but induces IFN in primary cells and in vivo (Mayr et al. 1975), and (v) the remarkable efficacy of recombinant MVA vaccines tested in various animal models for infectious diseases and cancer (Sutter et al., 1994, Hirsch, et al. 1996, Carroll, et al. 1997, Schneider et al., 1998).

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An HLA-A2 Polyepitope Vaccine for Melanoma Immunotherapy[1]

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Epitope-based vaccination strategies designed to induce tumor-specific CD8 CTL are being widely considered for cancer immunotherapy. Here we describe a recombinant poxvirus vaccine that codes for ten HLA-A2-restricted epitopes derived from five melanoma Ags conjoined in an artificial polyepitope or polytope construct. Target cells infected with the melanoma polytope vaccinia were recognized by three different epitope-specific CTL lines derived from HLA-A2 melanoma patients, and CTL responses to seven of the epitopes were generated in at least one of six HLA-A2-transgenic mice immunized with the construct. CTL lines derived from vaccinated transgenic mice were also able to kill melanoma cells in vitro. Multiple epitopes within the polytope construct were therefore shown to be individually immunogenic, illustrating the feasibility of the polytope approach for melanoma immunotherapy. Tumor escape from CTL surveillance, through down regulation of individual tumor Ags and MHC alleles, might be overcome by polytope vaccines, which simultaneously target multiple cancer Ags. *The Journal of Immunology,* 1999, 163: 4058–4063.

A common feature of malignant melanoma is the expression of multiple Ags, which are recognized by $\alpha\beta$ CD8$^+$ CTL. Recent human therapeutic vaccine trials, which utilize the epitopes recognized by such CTL, have illustrated the potential for CTL epitope-based immunotherapeutic vaccine strategies (1, 2). Such strategies do not require surgical removal and culture of autologous tumor cells from the patient, and the use of autologous dendritic cells might also be avoided if effective, safe vaccine vectors can be developed (3). CTL epitope-based approaches offer a number of potential advantages over whole Ag-based cancer vaccines: 1) they can focus immunity toward optimal (4) and/or cryptic protective epitopes (5); 2) sequences that have oncogenic activity (6) or contain targets for autoimmune CD4 cells (7) are omitted; and 3) sequences that are the target of preexisting CD4 T cells or B cell responses are avoided. Such preexisting responses have the potential to deviate (8, 9) or inhibit (10, 11) effective CTL induction by a therapeutic vaccine.

Single epitope-based approaches have the disadvantage that an HLA-restricted CTL response can be raised to only one Ag. CTL responses specific for multiple Ags and restricted by multiple HLA alleles would clearly be desirable for cancer immunotherapy, given the variable expression of tumor Ags (12, 13) and MHC alleles

(14) by melanomas and their metastases. Targeting multiple Ags and MHC alleles might be achieved by using multiple recombinant Ags or mixtures of synthetic peptide epitopes. The former loses the advantages of epitope-based approaches and would require complex recombinant vaccine Ag mixtures or constructs. The latter is complicated by adjuvant considerations and by problems associated with peptide solubility, chemical modifications of certain amino acids, and interpeptide interactions (15). Here we describe the construction and testing of a melanoma polyepitope or polytope poxvirus vaccine that contains ten conjoined minimal HLA-A2-restricted CTL epitopes, derived from five melanoma Ags, in a single recombinant construct. Despite the large number of epitopes restricted by the same allele, multiple epitopes within the vaccine construct were either recognized by epitope-specific CTL from melanoma patients and/or generated epitope-specific CTL in HLA-A2-transgenic mice. The polytope approach thus allows multiple Ags to be simultaneously targeted and should increase a patient's spectrum of antitumor CTL responses.

Materials and Methods

Construction of the melanoma polytope recombinant vaccinia

A synthetic oligonucleotide fragment (see Fig. 1) was constructed from two 70-mer and four 67-mer synthetic oligonucleotides using Splicing by Overlap Extension and PCR (16). The nucleic acid sequence of the fragment coded for (from the 5' end) a cap, a BamHI restriction site, a Kozac sequence, a methionine start codon, 10 contiguous minimal melanoma CTL epitopes (see Table 1), a stop codon, and a SalI site and a cap at the 3' end. The amino acid sequences of the CTL epitopes were converted to DNA sequence using universal codon usage but were designed to avoid inclusion of unwanted restriction sites. Dimers were made of synthetic oligonucleotides 1 and 2 (reaction A), 3 and 4 (reaction B), and 5 and 6 (reaction C) (0.4 μg of each) in 40-μl reactions containing standard 1× *Pfu* PCR buffer, 0.5 mM dNTPs, and 1 U of cloned *Pfu* DNA polymerase (hot start at 94°C), using the thermal program 94°C for 10 s, 52°C for 20 s, and 72°C for 20 s for five cycles. At the end of 5 cycles, the PCR program was paused at 72°C, and 20-μl aliquots of the dimer reactions A and B were mixed (reaction C was left in the PCR machine) and subjected to a further 5 cycles (94°C for 10 s, 58°C for 20 s, and 72°C for 20 s). At cycle 10, the program was paused again; 20 μl of reaction C was added to 20 μl from the A + B mix; and a further 5 cycles was completed (94°C for 10 s, 52°C

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for 20 s, and 72°C for 20 s). Two 20-mer oligonucleotides (matching the first and last 20 bp of the sequence shown in Fig. 1) were used to PCR amplify the gel purified full-length product using the reaction mixed above at an annealing temperature of 52°C for 25 cycles. The full-length gel-purified PCR fragment was cloned into the EcoRV site of pBluescript II KS⁻. A correct DNA insert was cloned behind the vaccinia P7.5 promoter in the plasmid shuttle vector pBCBO6 using BamHI/SalI restriction enzymes. Construction of a TK⁻ recombinant virus was then conducted using marker rescue recombination as described previously (16, 17), generating the recombinant melanoma polytope (rVV.mel.pt)³ coding for 10 HLA-A2 melanoma epitopes (see Table I).

Human CTL lines

HLA-A2-positive patients, P5 and P11, had confirmed cutaneous malignant melanoma and were enrolled in a therapeutic vaccination trial at the Ludwig Institute Oncology Unit (18). CTL lines specific for AAGIGILTV and YLEPGPVTA were established from PBMC by sensitizing half the PBMC with peptide (Chiron Technologies, Clayton, Australia; or made in house at Queensland Institute of Medical Research (QIMR)) (10 µg/ml, 2 h, 37°C followed by two washes) and adding back to the remaining cells in a 24-well plate. The cells were cultured in RPMI 1640 media supplemented with 10% FCS (QIMR), 2 mM glutamine (ICN Biomed, Aust. Pty., Seven Hills, Australia), 100 µg/ml streptomycin, and 100 IU/ml penicillin (CSL, Melbourne, Australia), and 1 ml of medium containing 5 U/ml recombinant human IL-2 (kindly provided by Cetus, Emeryville, California) was added on day 3. On day 7, IL-2 and peptide were added to a final concentration of 25 U/ml and 1 µg/ml, respectively. Partial medium changes with 25 U/ml IL-2, but no peptide, were given as necessary. On day 14, the cultures were used as effectors in standard chromium release assays.

The LLDGTATLRL-specific line was generated by restimulation of PBMC (derived from leukapheresis) from patient A02, with the autologous irradiated (8000 rad) A02-Mb melanoma cells (two times, 7 days apart), followed by two restimulations (7 days apart) with peptide-sensitized (10 µg/ml, 37°C, 1 h), washed, irradiated (8000 rad) HLA-A2 lymphoblastoid cell lines (LCLs) (responder to stimulator ratio throughout, 20:1). IL-2 (25U/ml) was added on day 7, and the effectors were used on day 35.

Human target cells for murine and human CTL

An EBV (B95.8)-transformed LCL from a homozygous HLA-A2 healthy individual (HLA-A2⁺ LCL) was 1) infected with rVV.mel.pt or a control vaccinia recombinant vaccinia expressing an unrelated polytope construct (rVV.Cont.) (16) (multiplicity of infection 10:1) overnight, before ⁵¹Cr labeling, or 2) sensitized with peptide (10 µg/ml) at the same time as ⁵¹Cr labeling. The following cell lines were also used in standard 6-h ⁵¹Cr release assays: ATCC HTB-73 (HLA-A2-negative melanoma): ATCC HTB-64 (HLA-A2-positive melanoma) and HTB-102, a skin fibroblast line from the same patient: A02-Mb and A09-M, melanoma lines from HLA-A2-positive patients enrolled in a therapeutic clinical trial of GM-CSF-transduced autologous melanoma cells (C. Schmidt, M. O'Rourke, J. Parkes, M. Down, J. Bell, N. Block, R. Thomas, D. Thomas, B. Stafford, V. Nicholson, and K. Ellem, manuscript in preparation); A12-M, an HLA-A2-negative line from a patient in the same trial; and LAR1 (HLA-A2-positive) and ME235 (HLA-A2-negative), Mart-1-expressing melanoma cell lines (18). HLA-A2⁺ LCLs were used as cold target inhibitors (80:1) for assays using human CTL derived from donors P5 and P11.

Vaccination and CTL assays using HHD transgenic mice.

Transgenic HHD mice have a transgene comprising the α1 and α2 domains of HLA-A2 linked to the α3, transmembrane, and cytoplasmic domains of H-2Dᵇ, with the α1 domain linked to human β₂-microglobulin. This transgene was introduced into murine β₂-microglobulin and H-2Dᵇ double knockout mice; thus, the only MHC expressed by the HHD mouse was the modified HLA-A2 molecule (19).

HHD mice were vaccinated i.p. with 1 × 10⁸ PFU recombinant vaccinia virus coding for the melanoma polytope (rVV.mel.pt) or a control polytope vaccinia coding for a series of EBV epitopes (17). Naive control mice animals were not vaccinated. After 3 wk, splenocytes were harvested, and 5 × 10⁶ cells were restimulated in 24-well plates with 1 × 10⁶ LPS blasts

³ Abbreviations used in this paper: rVV.mel.pt, recombinant vaccinia virus expressing the melanoma polytope; rVV.Cont., recombinant vaccinia virus expressing a control polytope construct; HHD mice, transgenic mice expressing α1 and α2 of HLA-A2 linked to the α3 and transmembrane and cytoplasmic domains of H-2Dᵇ and linked to human β₂-microglobulin carried on a murine β₂-microglobulin and H-2Dᵇ double knockout background; LCL, lymphoblastoid cell line; EL4S3⁻RobHHD, murine β₂-microglobulin-deficient EL4 cells transfected with the HHD transgene.

Table I. *HLA A2-restricted melanoma epitopes included in the melanoma polytope construct*

Melanoma Ag	Peptide Sequence	References
Mart-1 (27-35)	AAGIGILTV	46
MAGE-3 (271-279)	FLWGPRALV	47
Tyrosinase (1-9)	MLLAVLYCL	48
gp100 (457-466)	LLDGTATLRL	49
gp100 (154-162)	KTWGQYWQV	25
Tyrosinase (368-376)	YM (N/Dᵃ) GTMSQV	48
gp100 (209-217)	ITDQVPFSV	50
gp100 (280-288)	YLEPGPVTA	50
Mart-1 (32-40)	ILTVILGVL	51
N-acetylglucosaminyl-transferase V gene intron	VLPDVFIRCV	52

ᵃ Position 370 is an N in the native protein but the epitope recognized by CTL has a D (generated by deamidation) in this position (53). The polytope codes for D in this position.

(20), which were sensitized with peptide (10 µg/ml for 1 h at 37°C), irradiated (8000 rad), and washed twice. Cells were cultured in RPMI 1640 media supplemented with 10% FCS (QIMR), 2 mM glutamine (ICN), 5 × 10⁻⁵ M β-mercaptoethanol (Sigma, St. Louis, MO), 100 µg/ml streptomycin, and 100 IU/ml penicillin (CSL). On day 4, 1 ml of medium was added containing 5 U/ml recombinant human IL-2 (Cetus) or rat lymphocyte IL-2 (ICN). On day 6, the cultures were used as effectors in standard 6 h ⁵¹Cr release assays against 1) human cell lines (see above) and 2) EL4S3⁻RobHHD cells (19), which were sensitized with the indicated peptide (10 µg/ml) at the same time as radio-labeling and washed twice before use. All CTL assays were performed in duplicate for each E:T ratio. Further weekly restimulations in vitro were performed on some cultures by using peptide-sensitized, gamma-irradiated (8000 rad) EL4S3⁻RobHHD cells as stimulators (effector:stimulator ratio 20:1).

Results

A recombinant melanoma polytope vaccinia virus (rVV.mel.pt) was constructed that coded for ten conjoined HLA-A2 melanoma epitopes (Table I). The artificial recombinant insert (Fig. 1) was generated by using synthetic oligonucleotides and PCR. The DNA and protein sequence of the melanoma polytope construct is shown in Fig. 1.

Melanoma-specific CTL lines recognized the melanoma polytope construct

Three epitope-specific CTL lines from three melanoma patients (P5, P11, and A02) were generated and were shown to be specific for AAGIGILTV, YLEPGPVTA, and LLDGTATLRL by their ability to lyse HLA-A2⁺ LCLs sensitized with each peptide, respectively (Fig. 2, HLA-A2⁺ LCL⁺/⁻ peptide). The melanoma specificity of the CTL lines from donors P5 and P11 was illustrated by their ability to recognize HLA-A2⁺ melanoma lines (LAR1 and HTB64), but not HLA-A2-negative melanoma lines (ME235 and A12-M) or, in the case of P11, a fibroblast line (HTB-102) derived from the same individual as the HTB-64 melanoma line (Fig. 2, melanoma lines). (Although donor A02 had CTL reactivity against LLDGTATLRL, the melanoma line A02-Mb derived from the bowel metastasis of this patient did not appear to present gp100; data not shown).

Each of the epitope-specific CTL lines was capable of recognizing LCLs infected with rVV.mel.pt (Fig. 2; HLA-A2⁺ LCL⁺ rVV.mel.pt), but not a control rVV (Fig. 2; rVV.Cont.), illustrating that each of these three epitopes was individually processed from the melanoma polytope construct and presented to melanoma-specific CTL.

POLYTOPE VACCINE FOR MELANOMA IMMUNOTHERAPY

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CAP BamH1 Kozak Start

```
ata gga tcc acc atg gct gct ggt atc ggt atc ctg acc gtt
 I   G   S   T   M   A   A   G   I   G   I   L   T   V

ttc ctg tgg ggt ccg cgt gct ctg gtt atg ctg ctg gct gtt ctg tac tgc ctg
 F   L   W   G   P   R   A   L   V   M   L   L   A   V   L   Y   C   L

ctg ctg gac ggt acc gct acc ctg cgt ctg aaa acc tgg ggt cag tac tgg cag gtt
 L   L   D   G   T   A   T   L   R   L   K   T   W   G   Q   Y   W   Q   V

tac atg gac ggt acc atg tcc cag gtt atc acc gac cag gtt ccg ttc tcc gtt
 Y   M   D   G   T   M   S   Q   V   I   T   D   Q   V   P   F   S   V

tac ctg gaa ccg ggt ccg gtt acc gct atc ctg acc gtt atc ctg ggt gta cta
 Y   L   E   P   G   P   V   T   A   I   L   T   V   I   L   G   V   L

gtt ctg ccg gac gtt ttc atc cgt tgc gtt tag gtc gac cgt
 V   L   P   D   V   F   I   R   C   V   *   V   D   R
                                           Stop SalI  CAP
```

FIGURE 1. Nucleotide and amino acid sequence of the melanoma polytope construct. The first and then every second CTL epitope are underlined.

Mice vaccinated with the melanoma polytope generated CTL specific for multiple epitopes

To determine whether the polytope construct was capable of raising CTL responses in vivo, HHD-transgenic mice were vaccinated with the rVV.mel.pt. CTL responses were generated to AA GIGILTV, LLDGTATLRL, KTWGQYWQV, YMDGTMSQV. ITDQVPFSV, YLEPGPVTA, and VLPDVFIRCV (Fig. 3). Not all the mice tested generated response to all the epitopes; five of the 6 (5/6) mice vaccinated with the rVV.mel.pt generated responses to AAGIGILTV, 2/6 mice tested generated responses specific for LLDGTATLRL, 1/7 for KTWGQYWQV, 3/6 for YMDGTMSQV, 2/6 for ITDQVPFSV, 2/6 for YLEPGPVTA, and 6/7 for

VLPDVFIRCV. Fig. 3 shows the average lysis of CTL effectors generated from responder mice, which were defined as mice with effector populations giving peptide-specific lysis of more than 10%. None of the mice tested generated CTL specific for FLWG-PRALV, MLLAVLYCL, and ILTVILGVL following rVV.mel.pt immunization (Fig. 3). The total number of mice tested for these epitopes was 13, and Fig. 3 illustrates the mean lysis values for all these effector populations ($n = 13$ for each). Immunization of HHD mice with FLWGPRALV, MLLAVLYCL, and ILTVIL GVL peptide-based vaccines also failed to induce CTL responses
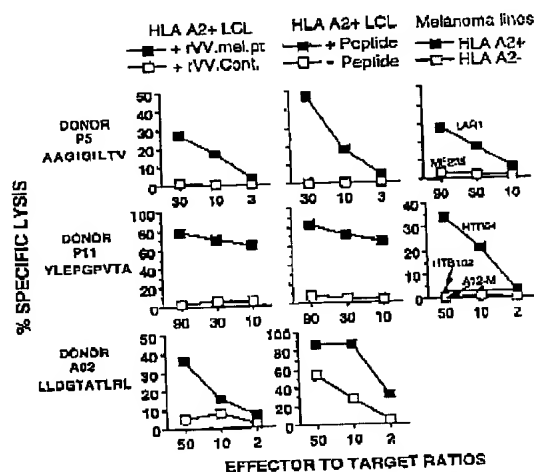
FIGURE 2. Epitope-specific CTL lines derived from PBMC donated by three melanoma patients were used as effectors against three different target cell types. First column, HLA-A2+ LCLs infected with the rVV.mel.pt (■) or a control rVV (□); second column, HLA-A2+ LCLs sensitized with the indicated peptide (i.e., AAGIGILTV for donor P5 and YLEPGPVTA for P11) (■) or the same LCL without peptide (□); and third column, melanoma cell lines expressing HLA-A2 (LAR1 and HTB64) (■) and control lines, which are HLA-A2 negative (ME235 and A12-M) (□). A fibroblast line HTB-102, derived from the same individual as the melanoma HTB-64, is shown as an extra negative control (▲).
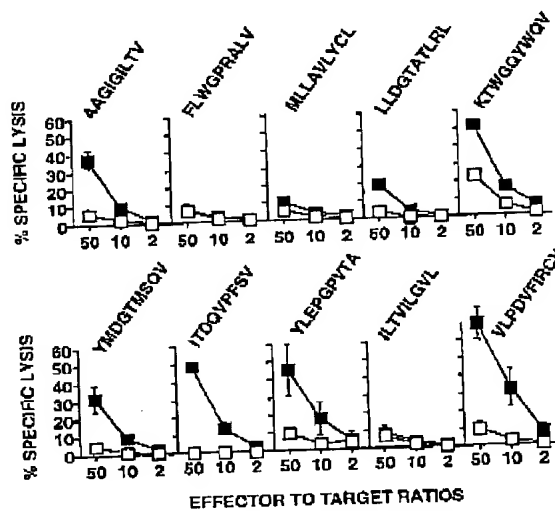
FIGURE 3. HHD mice were immunized with rVV.mel.pt. Splenocytes were separately restimulated in vitro with each of the indicated peptides and used as effectors against target cells sensitized with the same peptide (■) or no peptide (□). Mean lysis values (+SE) for responder mice are presented. Nonresponder mice for any given peptide were defined as animals whose three times restimulated effectors gave less than 10% peptide-specific lysis (calculated as the percentage specific lysis of target cells sensitized with peptide, minus the percentage specific lysis of target cells without peptide). For epitopes where no response was detected in any animals, the graphs show mean lysis values (+SE) of all mice tested.

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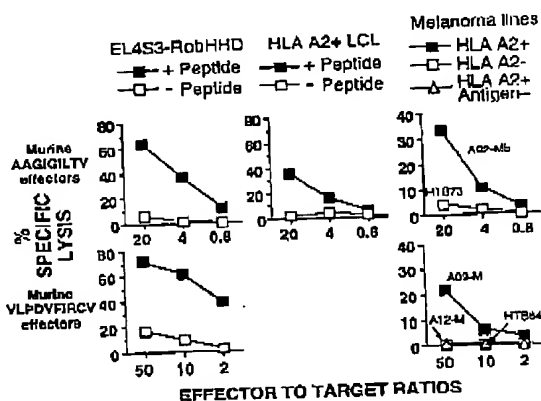
FIGURE 4. Bulk effectors from rVV.mel.pt-immunized mice were restimulated in vitro and used against (first column) EL4S3⁻RobHHD cells sensitized with peptide (■) or the same cells without peptide (□); (second column) HLA-A2⁺ LCLs sensitized with the indicated peptide (■) or the same LCL without peptide (□); and (third column) melanoma cell lines expressing HLA-A2 (A02-Mb and A09-M; ■) and control lines, which are HLA-A2 negative (HTB73 and A12-M; □) or are HLA-A2⁺ but do not present the target Ag (HTB64; △).

unless large amounts of peptide were used (100 μg per mouse) and the vaccine contained a source of codelivered CD4 T cell help.[4]

Splenocytes from 1) naive HHD mice and 2) HHD mice immunized with an unrelated recombinant vaccinia coding for an EBV CTL polytope (17) were also restimulated in vitro with the melanoma peptides. None of the effector populations generated from these animals (even after five weekly restimulations) were capable of lysing targets sensitized with the melanoma peptide used for restimulation (data not shown).

These data illustrated that the melanoma polytope vaccine was able to induce in vivo CTL responses specific for multiple HLA-A2 melanoma CTL epitopes.

rVV.mel.pt-induced CTL recognize human melanoma cells

The inability of the murine CD8 molecule to bind effectively to the α3 domain of human MHC means that lysis of human HLA-A2⁺ target cells by CTL from A2Kᵇ-transgenic mice tends to be poor (21). The same problem would be expected for HHD-derived effectors, which should also lyse HHD-transfected target cells more efficiently than HLA-A2-expressing cells (19). To overcome this problem and determine whether CTL effectors derived from rVV.mel.pt-immunized HHD mice would be capable of lysing HLA-A2⁺ melanoma cells, AAGIGILTV and VLPDVFIRCV effectors were subjected to three rounds of peptide restimulation in vitro. The resulting bulk effectors had high sp. act. for peptide-sensitized target cells expressing the HHD transgene (Fig. 4; EL4S3⁻RobHHD). Despite the CD8/α3 mismatch, these effectors were also capable of killing peptide-sensitized HLA-A2⁺ LCLs, although with the expected reduction in sp. act. (Fig. 4; HLA-A2⁻ LCL). Importantly, these effectors were able to lyse HLA-A2⁺ melanoma cell lines expressing the relevant tumor Ags (A02-Mb and A09-M), but not HLA-A2⁻ melanoma cell lines (HTB-73 and A12-M) or an HLA-A2⁺ melanoma cell line, which does not ex-

[a] H. Firat, F. Garcia-Pons, S. Tourdot, S. Pascolo, A. Scardino, Z. Garcia, M.L. Michel, R.W. Jock, G. Jung, K. Kosmatopoulos, et al. 1999. H-2 class I knockout, HLA A2.1-transgenic mice: a versatile animal model for preclinical evaluation of anti-tumour immunotherapeutic strategies. *Eur. J. Immunol. In press.*

press the target Ag (HTB-64) (Fig. 4; melanoma cells). These data illustrated that the melanoma polytope vaccine had induced CTL capable of recognizing melanoma Ags processed and presented by human melanoma cells.

Discussion

This paper illustrates the feasibility of delivering multiple HLA-A2-restricted melanoma CTL epitopes using the polytope vaccination strategy. The melanoma polytope vaccine induced epitope-specific CTL of multiple specificities in HLA-A2-transgenic mice and was recognized by CTL lines from HLA-A2 melanoma patients, arguing that multiple epitopes from the melanoma polytope vaccine can be simultaneously processed and presented. Polytope vaccine-induced CTL were also able specifically to kill human melanoma cells, suggesting that polytope vaccination can induce CTL of sufficient affinity to kill physiologically relevant target cells. This is likely to be a critical feature for cancer vaccines, given the down-regulation of HLA by melanoma cells (14) and the potential for induction of low affinity CTL by peptide vaccination (22, 23).

The HHD mouse system represents a useful model for preclinical and quality control testing of vaccines designed to induce HLA-A2-restricted CTL responses in humans. However, as reported previously, HLA-A2-transgenic mice appear unable to respond to some known HLA-A2-restricted epitopes (24). In this study, HHD mice failed to respond to FLWGPRALV, MLLAV LYCL, and ILTVILGVL following rVV.mel.pt immunization. In addition, variable induction of CTL specific for some epitopes was also observed in individual transgenic mice (Ref. 24; Fig. 3 legend). These deficiencies may reflect 1) a limited and variable TCR repertoire in HLA-transgenic mice (discussed below) and/or 2) the poor immunogenicity of individual epitopes. MLLAVLYCL and ILTVILGVL bind poorly to HLA-A2.1,[4] and polytope vaccination may provide insufficient amounts of these epitopes to promote efficient priming. The HLA-A2.1 binding and immunogenicity of ITDQVPFSV, KTWGQYWQV. and YLBPGPVTA peptides have been improved by changing the anchor residues to IMDQVPFSV, KLWGQYWQV. and YLEPGPVTV (2, 25, 26). A polytope vaccine's ability to prime responses to poorly immunogenic epitopes might be improved if such epitopes were replaced with anchor-modified epitopes, which have higher HLA-A2-binding affinities.

As noted previously (24), a contributing factor to 1) the inability of HLA-A2-transgenic mice to respond to some HLA-A2 epitopes, and 2) the variable responses seen with other epitopes may be a limited and variable TCR repertoire educated on the HLA-A2 transgene in these animals. Murine TAP proteins appear to be more selective than their human equivalents (27), and other murine proteins involved in processing and presentation may also be inefficient at delivering some peptides for HLA binding (28, 29). Although these factors may result in the inefficient processing and presentation of some vaccine Ags, the inability of all HLA-A2 peptide epitope immunogens to induce efficiently CTL responses in all HLA-A2-transgenic mice[4] (24) suggests that the main problem may be a restricted and variable TCR repertoire. A reduced quantity and/or diversity of self epitopes loaded onto the A2/Kᵇ or HHD transgene in the thymus will limit positive selection of HLA-A2-restricted CTL, which is likely to limit the diversity of the HLA-A2-restricted TCR repertoire in the periphery of these animals (30). The intermouse variation in responses to some epitopes may reflect a heterogeneous TCR repertoire, which could arise from minor histocompatibility differences between individual HHD mice (19). Negative selection or deletion of CTL (as opposed

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to lack of positive selection) by murine equivalents of the melanoma epitopes is unlikely to be responsible for the inability of HHD mice to respond to certain epitopes. The sequence of the murine equivalent of FLWGPRALV is FLWGPRAHA and of MLLAVLYCL is MFLAVLYCL; thus, both murine homologues have changes in the anchor residues (underlined), which should prevent efficient binding to HLA-A2 (24). The ITDQVPFSV epitope, to which a response was generated, is equivalent in the mouse and the human gp100 melanocyte protein. However, autoimmunity against melanocytes could not be readily detected (31, 32) in HHD mice since the HHD transgene was integrated in the vicinity of the SJL-mouse's mutated tyrosinase gene, so all HHD mice are albinos (19).

A potentially important question for future polytope cancer vaccines is the source of CD4 T cell help. Should the vaccine induce CD4 helper responses specific for tumor Ags (33) or might vaccine induced CD4 help best be obtained from unrelated Ags (1, 34)? CD4 help is often required for optimal CTL induction but is also likely to be required for the maintenance of ongoing antitumor CTL responses (35). A virus-vectored melanoma polytope vaccine (like rVV.mel.pt) would induce CD4 responses specific for viral Ags and would not induce, or rely on, melanoma-specific CD4 responses. This may actually be advantageous in a clinical setting if the patients' tumor-specific CD4 T cell responses are deleted (36), anergized (37), or Th2 deviated (8, 9) by the tumor. In contrast, vaccine-induced melanoma-specific CD4 responses may synergize with vaccine-induced CTL, resulting in improved antitumor responses (33). Apoptotic tumor cells killed by vaccine-induced CTL are also likely to induce tumor-specific CD4 responses (38), which may also influence vaccine-induced antitumor CTL.

As more melanoma Ags and target epitopes are identified, a panel of polytope vaccines might be envisaged, with each vaccine containing multiple epitopes restricted by one HLA allele. An appropriate HLA-matched mixture might be then delivered to cover all the HLA alleles expressed by any individual patient. Downregulation of some or all HLA alleles by the melanoma cells should increase their susceptibility to NK/LAK lysis (39). A variety of delivery modalities might be used for human melanoma polytope vaccines; these include attenuated poxvirus vectors (40), adenovirus (41), naked DNA (42), or transfected dendritic cells (43). CTL induction might also be enhanced by codelivery of cytokines (44, 45) and/or prime boost strategies (40).

Acknowledgments

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Immunogenicity of a Human Immunodeficiency Virus (HIV) Polytope Vaccine Containing Multiple HLA A2 HIV CD8+ Cytotoxic T-Cell Epitopes

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Compelling evidence now suggests that αβ CD8 cytotoxic T lymphocytes (CTL) have an important role in preventing human immunodeficiency virus (HIV) infection and/or slowing progression to AIDS. Here, we describe an HIV type 1 CTL polyepitope, or polytope, vaccine comprising seven contiguous minimal HLA A2-restricted CD8 CTL epitopes conjoined in a single artificial construct. Epitope-specific CTL lines derived from HIV-infected individuals were able to recognize every epitope within the construct, and HLA A2-transgenic mice immunized with a recombinant virus vaccine coding for the HIV polytope also generated CTL specific for different epitopes. Each epitope in the polytope construct was therefore processed and presented, illustrating the feasibility of the polytope approach for HIV vaccine design. By simultaneously inducing CTL specific for different epitopes, an HIV polytope vaccine might generate activity against multiple challenge isolates and/or preempt the formation of CTL escape mutants.

A considerable body of compelling indirect evidence suggests that cytotoxic T lymphocytes (CTL) have a role in preventing or limiting (i) initial human immunodeficiency virus (HIV) infection (36) and (ii) progression to AIDS (16). Correlations between CTL activity and protection against challenge have been observed in lentivirus models (12, 22) and in studies of HIV-exposed but uninfected individuals (36). The inverse correlation between viral load and CTL levels in HIV patients also implies a significant role for HIV-specific CTL in the control of HIV replication (31). Direct evidence for the importance of CTL was recently obtained from an ovine retrovirus model in which a prophylactic vaccine designed to induce only CTL, prevented the establishment of a latent infection (21).

Induction of protective HIV-specific CTL responses is complicated by the presence of multiple HIV variants, any one of which may contain mutations in the target CTL epitopes (16), and/or by CTL escape mutants being rapidly generated following infection (16, 29). An ideal vaccine might induce a sufficient diversity of CTL specificities to ensure CTL-mediated protection against all or most of the potential variants within HIV challenge inocula and perhaps also preempt the generation of CTL escape mutants. Vaccines containing multiple recombinant antigens (10) may be able to induce CTL populations sufficiently diverse to be capable of cross-recognizing multiple isolates (15); however, even if homology sufficient to make such an approach feasible existed, highly variant epitopes may dominate at the expense of relatively conserved, protective subdominant epitopes (30). A CTL epitope-based approach has the advantage of being able to focus immunity toward protective, perhaps less variant, epitopes. Sequences outside the CTL epitope regions, which might adversely affect the immune response (7, 17, 20), can also be avoided. However, an epitope-based approach would be of advantage only if multiple CTL epitopes covering a range of epitopes could be simultaneously codelivered to induce a defined spectrum of CTL specificities. The polyepitope, or polytope, approach represents a strategy whereby multiple contiguous minimal CTL epitopes can be delivered as a single artificial construct (1, 14, 19, 38, 40, 41). Here, we demonstrate the immunogenicity of an HIV polytope vaccine containing multiple contiguous HLA A2-restricted HIV CTL epitopes from a range of HIV antigens. The vaccine construct was recognized by human HIV-specific CTL and raised multiple independent CTL responses in HLA A2-transgenic mice. Thus, apart from offering a considerable reduction in size compared to a recombinant multiantigen construct, the polytope approach represents an attractive strategy for CTL-based HIV vaccine design.

MATERIALS AND METHODS

HIV polytope and other recombinant vaccinia viruses. The HIV polytope recombinant vaccinia virus (rVV.HIV.pt) was constructed as follows. A synthetic oligonucleotide fragment (Fig. 1) was constructed from three 70-mer and one 72-mer synthetic oligonucleotides by the splicing-by-overlap-extension method and PCR (40, 41). The nucleic acid sequence of the fragment contained (from the 5' end) a BamHI restriction site, a Kozac sequence, a methionine start codon, sequences corresponding to seven contiguous minimal HLA A2 HIV CTL epitopes (Table 1), and a stop codon and a SalI site at the 3' end. The amino acid sequences of the CTL epitopes were converted to DNA sequences by using universal codon usage, but inclusion of restriction sites was avoided. Dimers were made of synthetic oligonucleotides 1-2 and 3-4 (0.4 µg of each) in 40-µl reaction mixtures containing standard 1× Pfu PCR buffer, 0.5 mM deoxynucleoside triphosphates, and 1 U of cloned Pfu DNA polymerase (hot start at 94°C) with the following thermal program: 94°C for 10 s, 52°C for 20 s, and 72°C for 20 s for five cycles. At the end of five cycles the PCR program was paused at 72°C and 20-µl aliquots of the two dimer reaction mixtures were mixed and subjected to a further five cycles (94°C for 10 s, 48°C 20 s, and 72°C for 20 s). The reaction

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FIG. 1. HIV polytope insert. The first epitope and then every second CTL epitope are underlined.

mixture was resolved on a 3% agarose gel, the 220-bp fragment was excised, and the agarose was removed by microcentrifugation through filter paper. Two 20-mer oligonucleotide primers (Fig. 1) were used to amplify by PCR the full-length product for 25 cycles at an annealing temperature of 50°C. The full-length gel-purified PCR fragment was cloned into the EcoRV site of pBluescript II KS(−) and checked by sequencing. The insert was then subcloned behind the vaccinia virus p7.5 promoter in the plasmid shuttle vector pPS 7.5 A with BamHI and SalI. Construction of the recombinant vaccinia virus was then performed by marker rescue recombination as described previously (8), by insertion of the fragment into the F region of a thymidine kinase-negative vaccinia virus, followed by plaque purification and selection with methotrexate.

Recombinant vaccinia virus containing HIV nef and pol expressed p27 Net, and the reverse transcriptase and integrase of the LAI strain of HIV (Transgene, Strasbourg, France) were made available via the Programme Reactifs de L'ANRS. The control vaccinia virus, rVV.Cont, coded for ovalbumin or the murine polytope (40).

PCR and RT-PCR of rVV-HIV-pt-infected cells. CV1 cells (2.5 × 10⁶) were infected with rVV.HIV.pt (multiplicity of infection = 5) and stored overnight, and the RNA was extracted and reverse transcribed as described previously (26). A control sample was also prepared without reverse transcriptase (RT). DNA was extracted from a parallel culture of rVV.HIV.pt-infected CV1 cells with a blood kit (Qiagen, Hilden, Germany). PCR was performed in a 20-μl volume containing 1 μl of cDNA or DNA (or an equivalent volume of the control samples) and 0.5 μl each of forward and reverse 20-bp primers (Fig. 1) (20 μM). The reaction mixture was as described previously (26). An initial denaturation at 95°C for 2 min was followed by 30 cycles of PCR (95°C for 10 s, 55°C for 10 s, and 72°C for 50 s) and a 10-min extension at 72°C. PCR products were resolved on a 2% agarose gel. The ~220-bp fragments were excised, purified (Wizard purification kit; Promega, Madison, Wis.), and sequenced.

Human CTL lines. Blood was obtained from HIV-infected patients at the Infectious Diseases Unit, Royal Brisbane Hospital. Most of the patients were receiving highly active antiretroviral therapy (HAART) at the Infectious Diseases Unit. HLA A2-positive individuals were identified by fluorescence-activated cell sorter analysis of peripheral blood mononuclear cells (PBMC) with an HLA A2-specific mouse monoclonal antibody derived from the supernatant of the hybridoma line ATCC HB72, BB7.2, followed by fluorescein isothiocyanate-labelled anti-mouse F(ab')₂ (Silenus, Melbourne, Australia). Cells were fixed in fresh 2% paraformaldehyde in phosphate-buffered saline prior to analysis.

PBMC from HLA A2 HIV-infected individuals were prepared by standard Ficoll-Paque gradient separation, and half of the cells were sensitized with the peptides indicated below (see Fig. 3 and Table 2) (Chiron Technologies, Clayton, Australia) (50 μg/ml, 1 h at 37°C, followed by one wash). The sensitized PBMC were added back to the remaining cells in a 24-well plate to 1 × 10⁶ to 2 × 10⁶ cells/ml (effector-to-stimulator ratio, 1:1). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (QIMR), 2 mM glutamine (ICN Biomed, Aust. Pty. Ltd., Seven Hills, Australia), and 100 μg of streptomycin per ml and 100 IU of penicillin per ml (CSL Ltd., Melbourne, Australia). Interleukin-7 (IL-7) (300 IU/ml; Sigma, St. Louis, Mo.) was added on day 0, and IL-2 (10 IU/ml; kindly provided by Cetus Corp., Emeryville, Calif.) was added on day 3. The bulk effectors were used in standard 6-h ⁵¹Cr release assays on days 10 to 12, unless stated otherwise. Partial medium changes were performed when required. Some cultures were maintained by weekly restimulations with HLA A2 lymphoblastoid cells that were peptide sensitized (10 μg/ml, 37°C for 1 h, washed, and irradiated [8,000 rads]: responder-to-stimulator ratio = 20:1). Fewer than seven CTL bulk cultures (one for each peptide) were set up when PBMC were limiting, and for these cases, restimulation with SLYNTVATL was always included.

The target cell for the epitope-specific bulk CTL effectors was an Epstein-Barr virus (B95.8)-transformed lymphoblastoid cell line (LCL) from an unrelated homozygous HLA A2 healthy individual (HLA A2+ LCL). HLA A2+ LCLs were either (i) infected (multiplicity of infection, 10) overnight with rVV.HIV.pt, a recombinant vaccinia virus coding for the specified HIV antigen, a control recombinant vaccinia virus expressing ovalbumin, or an unrelated polytope con-

struct (rVV.Cont) (40) prior to ⁵¹Cr labelling or (ii) sensitized with peptide (10 μg/ml) at the same time as ⁵¹Cr labelling followed by two washes before use in the ⁵¹Cr release assays. HLA A2+ LCLs were used as cold target inhibitors at a cold-to-hot ratio of 40:1.

Vaccination and CTL assays with HHD transgenic mice. Transgenic HHD mice have a transgene comprising the α1 (H) and α2 (H) domains of HLA A2 linked to the α3 transmembrane and cytoplasmic domains of H-2Dᵇ (D), with the α1 domain linked to human β₂ microglobulin. This transgene was introduced into murine β₂ microglobulin and H-2Dᵇ double knockout mice; thus, the only major histocompatibility complex (MHC) molecule expressed by the HHD mouse was the modified HLA A2 molecule (32).

For the first experiment, six HHD mice were vaccinated intraperitoneally with 5 × 10⁷ PFU of rVV.HIV.pt. After 3 weeks splenocytes were harvested and pooled, and 5 × 10⁶ splenocytes were restimulated with 1 × 10⁶ lipopolysaccharide blasts per 24-well plate (42). The lipopolysaccharide blasts were sensitized with peptide (10 μg/ml for 1 h at 37°C), irradiated (3,000 rads), and washed twice prior to use. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (QIMR), 2 mM glutamine (Sigma), 5 × 10⁻⁵ M β-mercaptoethanol (Sigma), and antibiotics as described above. On day 4, 1 ml of medium containing 5 IU of recombinant human IL-2 (Cetus) per ml was added. On day 6 the cultures were used as effectors in standard 6-h ⁵¹Cr release assays against EL4S3-Rob1HHD target cells (32), which were sensitized with the indicated peptide (10 μg/ml) at the same time as being radiolabelled and were washed twice prior to use.

For the DNA prime boost experiment mice were anesthetized with 100 μl of a solution containing ketamine (10 mg/ml), xylazine (2 mg/ml), and water (4:1:1) and were given 100 μg (50 μg into each quadriceps muscle) of pJWHIV (n = 3) or a control plasmid, pJW4303 (27) (n = 3) followed after 14 days with an identical booster injection. After another 14 days the mice received rVV.HIV.pt. Three weeks later the splenocytes were restimulated and ⁵¹Cr release was performed as described above, except that splenocytes from each animal were restimulated separately. Plasmid preparation was undertaken by using the EndoFree Plasmid Maxi kit (Qiagen). pJWHIV was generated by subcloning the HIV polytope insert from the HIV polytope pBluescript (see above) into pJW4303 (27) with HindIII and EcoRI.

RESULTS

Confirmation of the polytope sequence and transcription of the polytope insert. rVV.HIV.pt was constructed to contain a synthetic insert (Fig. 1) coding for seven HIV HLA A2 CTL epitopes (Table 1). The epitopes were selected from the list of optimal HLA A2 CTL epitopes described by Brander and Walker (2), excluding the more variant epitopes from env, and they included the relatively conserved gp120 epitope described by Dupuis et al. (9).

Direct sequencing of the insert in rVV.HIV.pt was used to confirm the presence of an uncorrupted polytope insert in the recombinant vaccinia virus. PCR of viral DNA extracted from rVV.HIV.pt-infected cells generated an ~220-bp fragment (Fig. 2, lane E), the expected size of the insert (Fig. 1). A water control for the PCR of viral DNA is shown in Fig. 2, lane D. Sequencing of the ~220-bp fragment gave the expected nucleotide sequence, shown in Fig. 1.

Polytope proteins have been very difficult to detect with antibody probes, possibly due to their lack of structure and resulting poor stability (40). RT-PCR was thus used to show appropriate transcription of HIV polytope mRNA by rVV.HIV.

TABLE 1. HLA A2-restricted HIV epitopes in the polytope[a]

Epitope (positions)	Sequence
Nef (157–166)	PLTFGWCYKL
Pol (346–354)	VIYQYMDDL
Gag (77–85)	SLYNTVATL
Nef (180–189)	VLEWRFDSRL
Pol (476–484)	ILKEPVHGV
gp120 (120–128)	KLTPLCVTL
Nef (190–198)	AFHHVAREL

[a] Epitopes were selected from those described by Brander and Walker (2) and Dupuis et al. (9). The more variable epitopes from env were not included.

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FIG. 2. Agarose gel of HIV polytope PCR products from cDNA (lane H) and viral DNA (lane E) derived from rVV.HIV.pt-infected cells. Lanes A and F, 1-kb markers (Gibco BRL, Gaithersburg, Md.); lane B. RT-PCR of the HIV polytope mRNA; lane C, RT-PCR control without RT; lane D, PCR control with water as template; lane E, PCR of viral DNA. Molecular size markers indicated by arrowheads are (from the top) 394, 344, 298, and 220 bp.

pt-infected cells (Fig. 2, lane B). A control for the RT-PCR without RT is shown in Fig. 2, lane C.

Following infection, rVV.HIV.pt thus transcribed an HIV polytope mRNA coding for seven HIV CTL epitopes.

Epitope-specific CTL lines from HIV patients recognize the HIV polytope construct. CTL from 14 HLA A2 HIV patients (Table 2) were separately restimulated in vitro with up to seven peptide epitopes (Table 1) to generate epitope-specific bulk CTL cultures. CTL cultures capable of recognizing at least one of the seven epitopes were generated from seven HLA A2 HIV patients (Fig. 3; Table 2). CTL cultures specific for more than one epitope were derived from the PBMC of five patients (Fig. 3; Table 2), with PBMC from H10 generating cultures specific for all seven epitopes (Fig. 3). The lower number of patients responding to SLYNTVATL in this study than in previous studies (3) may reflect the fact that nearly all the patients in this study were on HAART therapy.

The peptide-restimulated bulk cultures from the remaining seven HIV patients failed to generate significant peptide-specific activity; however, only three peptides could be tested for five of these patients (Table 2). An example of the data derived

from seven negative bulk cultures of the PBMC of one such individual, H28, is shown to illustrate the specificity of the in vitro restimulation protocol (Fig. 3). Two HLA A2 HIV-seronegative controls (HC1 and HC2) and three HIV-seropositive non-HLA A2 individuals (H12, H16, and H22) (Table 2) gave results essentially similar to those for H28 (data not shown), further demonstrating the specificity of the peptide restimulation protocol. Failure to generate epitope-specific CTL lines does not mean that the individuals did not have CTL specific for the corresponding antigen. Most HIV patients have CTL responses to at least one, and usually multiple, antigens (36). Detection of such CTL would require the use of antigen or HIV restimulation protocols, rather than the peptide restimulation used in this study.

The CTL effectors, which showed lysis against peptide-sensitized target cells (Fig. 3), also lysed target cells infected with rVV.HIV.pt (Fig. 3), illustrating that each epitope in the HIV polytope was individually processed and presented. Furthermore, in cases where sufficient bulk effectors were available, the CTL lines were also shown to be able to lyse LCLs infected with recombinant vaccinia virus coding for the whole antigen from which each respective epitope was derived (Fig. 3).

HHD mice vaccinated with the HIV polytope generated CTL specific for multiple epitopes. To determine whether the HIV polytope construct was capable of raising CTL responses in vivo, HHD transgenic mice were vaccinated with rVV.HIV.pt, and the splenocytes were restimulated in vitro and used to kill peptide-sensitized target cells (Fig. 4A). CTL responses to SL YNTVATL, ILKEPVIIGV, KLTPLCVTL, and AFHHVAR EL were generated. CTL responses to the remaining epitopes could not be generated in these mice by rVV.HIV.pt immunization (Fig. 4A and data not shown). The data illustrated that the HIV polytope vaccine was able to induce in vivo CTL responses to multiple HLA A2 HIV CTL epitopes.

To determine whether polytope CTL responses could be enhanced by using strategies combining DNA priming and boosters with recombinant vaccinia virus, as described previ-

TABLE 2. Summary of PBMC donor characteristics and CTL response(s)[a]

Donor	HLA	Age (yr)	CD4 count (10^6)/liter	RNA copies/μl	Date of HIV infection	HIV stage	CTL response(s)
H07	A2	34	260	1×10^5	4/1992	II	1/3 (VLE)
H08	A2	50	200	<400	2/1984	II	0/3
H09*	A2	41	380	1.3×10^5	1985	II	0/3
H10	A2	34	330	8.2×10^3	1985	IV C1	7/7[b]
H11	A2	50	320	2×10^4	1989	IV C1	0/3
H17*	A2	44	700	1.1×10^4	2/1996	II	0/3
H18*	A2	47	610	1.2×10^5	1985	II	0/3
H19	A2	35	90	1.3×10^5	12/1993	IV C1	2/7 (PLT, VTY)
H21	A2	47	440	<400	1986	II	2/7 (SLY, KLT)
H24	A2	48	300	580	1988	II	1/7 (SLY)
H27	A2	48	440	5.3×10^3	1/1991	II	3/7 (SLY, VIY, ILK)
H28	A2	41	60	1.3×10^5	1994	IV C	0/7
H30	A2	65	250	<400	5/1997	IV C	0/7
H33	A2	35	510	1.5×10^3	2/1997	II	3/7 (SLY, KLT, AFH)
H12	Not A2	37	350	<400	1995	III	0/3
H16	Not A2	44	300	<400	9/1997	II	0/3
H22	Not A2	32	520	<400	9/1996	IV C1	0/1
HC1	A2	46		0			0/7
HC2	A2	37		0			0/7

[a] CTL response(s) is given as the number of epitopes for which specific CTL activity could be generated per the number of epitopes tested (first three amino acids of the epitope(s) for which a response was seen). Date of HIV infection is given as year or month/year. HIV staging is based on the Centers for Disease Control and Prevention's surveillance case definition for AIDS. Donors HC1 and HC2 are healthy HIV-seronegative controls. *, not on HAART therapy at the time that blood was taken. The HAART treatment unresponsiveness of this cohort was similar to that described previously for patients with a history of extended treatment (34).
[b] Responses to all seven epitopes shown in Fig. 3.

FIG. 3. Epitope-specific CTL lines derived from PBMC of HIV-infected individuals (H2A, H10, H19, H21, and H33) used as effectors against (i) HLA A2+ LCLs sensitized with the indicated peptide (black squares) or not sensitized (white squares) (columns headed "Peptide"), (ii) HLA A2+ LCLs infected with rVV.HIV.pt (black squares) or a control recombinant vaccinia virus (white squares) (columns headed "Polytope"), and (iii) HLA A2+ LCLs infected with rVV.nef or rVV.pol (black squares) or a control recombinant vaccinia virus (white squares) (column headed "Antigen"). The epitope listed on the left of each row was used to restimulate the bulk cultures, which were used to generate the data in that row. Bulk cultures from each individual were separately restimulated with the indicated peptide, split, and used against peptide and polytope and sometimes against whole antigen expressing target cells. A summary of patient data is shown in Table 2. The negative results, for patient H28, are shown to illustrate the specificity of the in vitro restimulation protocol.

ously for whole-antigen-based vaccines (24, 35, 37), mice were immunized with a DNA vaccine coding for the HIV polytope and were then given a booster with rVV.HIV.pt. No significant improvement in the responses to epitopes which failed to generate a response following rVV.HIV.pt immunization (Fig. 4A) was observed following prime boost vaccination (Fig. 4B). The responses to SLYNTVATL, ILKEPVHGV, KLTPLCV TL, and AFHHVAREL were, however, significantly enhanced (an average of 2.4-fold at an effector-to-target ratio of 10:1, P = 0.008) by prime boost strategies (Fig. 4B). CTL responses to polytope vaccines can therefore also be enhanced by DNA prime-plus-poxvirus boost strategies.

DISCUSSION

Here, we demonstrate the feasibility of delivering multiple HLA A2 HIV CTL epitopes with a polytope vaccine construct. Each epitope in the polytope construct was recognized by CTL lines from HIV patients, and the polytope vaccine induced multiple epitope-specific responses in HHD transgenic mice. The DNA prime-plus-virus vector boost strategy (24, 35, 37) also improved CTL responses when applied to polytope vaccines.

Potential competition and/or immunodominance phenomena (30, 45) did not appear to interfere significantly with simultaneous presentation of, and priming by, the multiple HLA A2 epitopes within the polytope construct. Factors intrinsic to the epitope, such as MHC binding affinity, can determine the immunodominance of an epitope (45). For instance, the immunodominant SLYNTVATL (3) binds well to HLA A2 (44), whereas the subdominant AFHHVAREL (3) binds poorly to HLA A2 (23). However, the subdominance of an epitope can often be ascribed to inefficient proteolytic liberation of the epitope from the full-length protein (45). Such processing constraints are less likely to operate for polytope proteins, since these proteins appear to be rapidly degraded (40, 41) and the epitopes in the polytope are not flanked by poorly cleaved glycine or proline residues (13, 38, 41). The subdominance of AFHHVAREL may in part also reflect inefficient processing, since it appears to be codominant when presented in a polytope construct (Fig. 4). An ability to mitigate against dominance effects and generate multiple codominant responses may emerge as an important attribute of polytope vaccines.

A controversy over the HLA A2 restriction of AFHHVA REL was recently reported, based on the inability of this epitope to bind to HLA A2 efficiently in in vitro binding assays (4, 23). Two HIV-infected HLA A2 individuals recognized the AFHHVAREL epitope in this study, and rVV.HIV.pt vaccination induced AFHHVAREL-specific CTL in HHD mice, supporting the original contention that this epitope is re-

FIG. 4. Data from HHD mice immunized with HIV polytope vaccines. (A) Mice were immunized with rVV.HIV.pt, and pooled splenocytes were restimulated in vitro with each of the indicated peptides and used as effectors against target cells sensitized with the same peptide (black squares) or not sensitized (white squares). (B) Mice were immunized with two injections, either of DNA vaccine coding for the HIV polytope followed by rVV.HIV.pt (DNA/rVV) or of a control DNA plasmid followed by rVV.HIV.pt (rVV). Splenocyte populations from each mouse were individually restimulated with each peptide and used as effectors against target cells sensitized with peptide and not sensitized (Thus, the first six bars represent restimulation and lysis with PLTFGWCYKL.) Results were calculated as follows: percent lysis of target cells sensitized with peptide − percent lysis of target cells not sensitized (= standard error). DNA vaccination alone produced only weak CTL responses ranging from 5 to 10% (data not shown).

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stricted by HLA A2 (18). The relatively conserved gp120 CTL epitope, KLTPLCVTL, was recognized by three patients, suggesting that this also is a commonly recognized HLA A2-restricted epitope (9).

The HHD mouse system clearly represents an ideal model for preclinical and quality control testing of vaccines designed to induce HLA A2-restricted CTL responses in humans. The inability of HLA A2-transgenic mice to respond to some epitopes has been reported previously (44) and may reflect a limited T-cell receptor (TCR) repertoire educated on the HLA A2 transgene in these animals (32). Murine TAP proteins are more selective than their human equivalents (28), and other murine proteins involved in processing may also be inefficient at delivering some peptides for HLA binding (5, 33). Such factors may result in inefficient processing of certain polytope epitopes but may also limit the diversity of self-epitopes loaded onto HLA A2 in the thymus in HHD mice. The latter would reduce the diversity of the peripheral TCR repertoire educated on HLA A2 (11). Other factors are clearly also involved, since deletion of murine MHC expression in HHD mice appears to increase the TCR repertoire over that found in A2K^h transgenic mice (32), which retain murine MHC (10a, 44).

A prophylactic HIV polytope vaccine might ultimately contain a series of epitopes covering the diversity of HLA alleles in any target population and might also contain a number of common epitope variants. In a therapeutic setting, a cocktail of four single HLA polytope vaccines might be used to cover the four HLA alleles of any given individual patient. Several human delivery modalities vectors might be envisaged for HIV polytope constructs, perhaps in conjunction with prime boost and/or cytokine codelivery strategies (25, 41). These vectors include DNA-based vaccination (41, 43), avipoxvirus (6, 24), and/or modified vaccinia virus Ankara (13, 19, 37, 39).

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PATENT

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EXHIBIT AS14:

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Design of a polyepitope construct for the induction of HLA-A0201-restricted HIV 1-specific CTL responses using *HLA-A*0201* transgenic, *H-2* class I KO mice

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*HLA-A*0201* transgenic, *H-2D^b/mouse β2-microglobulin* double-knockout mice were used to compare and optimize the immunogenic potential of 17 HIV 1-derived, HLA-A0201-restricted epitopic peptides. A tyrosine substitution in position 1 of the epitopic peptides, which increases both their affinity for and their HLA-A0201 molecule stabilizing capacity, was introduced in a significant proportion, having verified that such modifications enhance their immunogenicity in respect of their natural antigenicity. Based on these results, a 13-polyepitope construct was inserted in the pre-S2 segment of the hepatitis B middle glycoprotein and used for DNA immunization. Long-lasting CTL responses against most of the inserted epitopes could be elicited simultaneously in a single animal with cross-recognition in several cases of their most common natural variants.

1 Introduction

There is accumulating evidence that CD8+ αβ cytotoxic T lymphocyte (CTL) response is a major immune effector arm against HIV infection. The viremia of the primary infection decline with the onset of the CD8+ CTL response, weeks before the appearance of neutralizing antibodies [1–3]. The early development in certain individuals of CTL responses directed at multiple epitopes is associated with a slower disease progression, as well documented in infected newborns [4, 5]. In simian immunodeficiency virus infection of macaques, depletion of CD8+ T cells is immediately followed by an increase in viremia [6]. However, despite the development in most patients of potent CTL responses, in the absence of antiviral drug therapy, the infection usually inexorably progresses towards the AIDS stage, with some noticeable exceptions, in particular patients with the ΔCCR5 deletion [7].

In the natural course of the HIV infection, the development of cytolytic responses usually takes weeks, with differences among individuals depending in part on the HLA class I alleles they expressed. Noticeably, HLA-A0201 responses are significantly delayed, leaving time for the virus to hide as provirus and escape immune detection [8]. Since the initial viral burden is an important prognostic factor [9, 10], a more rapid onset of the appropriate CTL responses mediated by prophylactic vaccination should improve the prognosis of HIV 1 infection. Further support for the feasibility of a CTL-based vaccine approach comes from the observation that CTL memory is not dependent on continuous exposure to cognate antigens: continuous boosting would not be

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required for a prophylactic vaccine to mediate an improved prognosis [11–14].

During the last two decades, many laboratories have devoted themselves to the search of HIV-derived CTL epitopic peptides, in particular those presented by the HLA-A0201 molecules, encoded by the most prevalent HLA class I allele in the Caucasian population. Classical *HLA-A*0201* transgenic mice (which still express their own H-2 class I molecules) have been used to evaluate the immunogenic potential of identified epitopes and to compare different vaccine strategies [15, 16]. However, these transgenic mice develop CTL responses against a limited number of these epitopes. We recently created new strains of *HLA-A*0201* transgenic mice expressing a heavy chain fused by its N terminus to human β2-microglobulin (β2m) and in which both the *H-2D^b* and *mouse β2m* genes have been inactivated by homologous recombination [17]. These mice, hereafter designated HHD, express a single species of class I molecule detectable serologically (HLA-A0201) and generate a diversified HLA-A0201-restricted CTL response more efficiently than classical *HLA* transgenic mice [18]. Therefore, we used HHD mice (i) to compare the immunogenic potential of the HIV 1-derived CTL epitopes reported in the literature, (ii) to evaluate the possibility of modifying these epitopes to improve their immunogenicity without altering their antigenicity, (iii) to identify new potential HIV 1 epitopes and (iv) to test the capacity of a polyepitope construct to induce simultaneously in a single mouse CTL responses against several HIV 1-derived epitopes.

2 Results

2.1 Comparative immunogenicity of HIV 1-derived, HLA-A0201-restricted epitopic peptides

Fifteen demonstrated [19–27] and two potential [27] HIV 1-derived, HLA-A0201-restricted minimal epitopic peptides were selected from the literature (Table 1). Their immunogenicity was compared by vaccinating groups of six HHD mice with the peptides emulsified in IFA. Splenocytes were restimulated *in vitro* and the CTL activities evaluated in a ⁵¹Cr-release assay using HHD-transfected RMA-S target cells sensitized with relevant or control *Influenza* matrix (Inf.m.G9L) peptides.

The results are illustrated in Table 2. Considering the number of responder mice and the strength of their responses, the peptides were classified in strong (S9L, I9V), intermediate (T9V, L10V, A9M, V11V, V9L, L9V, K9L-

8S, R9V) or inefficient (E9V, P9L, E11Q, E10L, K9L, P10L, A9L) immunogens.

A similar evaluation was done by repeating the above experiment with co-injection of a I-A^b-restricted CD4 epitopic peptide (HBV core T13L), which was found in a previous work to enhance HLA-A0201-restricted CTL responses in HHD mice [18]. Whereas some responses were enhanced, some others (i.e. S9L) were abolished for unknown reasons. Globally, no significant improvement was observed (not shown).

2.2 Relative affinity for HLA-A0201 molecules and stabilizing capacities of the HIV 1-derived epitopic peptides

TAP-deficient T2 cells were incubated overnight with synthetic peptides and the amount and stability of cell surface HLA-A0201/peptide complexes were evaluated in an indirect immunofluorescence assay as described in detail elsewhere [28]. Relative affinities (RA; the ratio of the concentrations in the culture medium of the tested versus the reference peptide, I9V, resulting in equal amounts of cell surface-expressed peptide-associated HLA-A0201 molecules) and stabilizing capacities (DC50; the time following removal of the peptides from the culture for a 50% decay of cell surface stabilized HLA-A0201/peptide complexes) are given for each peptide in Table 3: the lower the RA, the higher the affinity of the tested peptide; the higher its DC50 value, the better its stabilizing capacity.

As a rule, the peptides able to induce CTL responses had high affinity and stabilizing capacity, the non-immunogenic peptides were both poor binders and poor stabilizers.

2.3 RA for HLA-A0201 molecules and stabilizing capacities of P1 tyrosine-substituted peptides

Substitution of the N-terminal (P1 position) residue by an aromatic amino acid (a favorable P1 secondary anchor residue for HLA-A0201 molecules [29]) has been reported to enhance the immunogenicity of the HIV 1 pol-derived I9V epitopic peptide (ILKEPVHGV to YLKEPVHGV) [30]. Similar improvement was demonstrated for a series of 30 HLA-A0201-restricted human cancer epitopic peptides [28]. We evaluated whether we could enhance the affinity and stabilizing capacity of the HIV 1-derived epitopic peptide by replacing the P1 amino acid with a tyrosine.

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Table 1. HIV I-derived CD8 epitopic peptides assayed

Protein of origin[a]		CD8 epitopic peptides	Frequency among HIV 1 isolates (%)[b]				[Ref]
			Clade A	Clade B	Clade C	All clades	
GAG	P17 (77–85)	SLYNTVATL (S9L)	56	46	33	32	[24]
	P24 (19–27)	TLNAWVKVV (T9V)	11	83	20	39	[25]
	P24 (212–221)	EMMTACQGV (E9V)[c]	89	97	100	93	[27]
POL	(79–88)	LLDTGADDTV (L10V)[c]	100	100	87	98	[27]
	(188–196)	ALVEICTEM (A9M)	0	79	0	21	[21]
	(263–273)	VLDVGDAYFSV (V11V)	89	90	93	56	[27]
	(334–342)	VIYQYMDDL (V9L)	33	93	87	78	[23]
	(464–472)	ILKEPVHGV (I9V)	11	76	80	61	[26]
	(576–584)	PLVKLWYQL (P9L)	89	100	93	86	[21]
	(669–679)	ESELVNQIIEQ (E11Q)	0	7	87	22	[21]
	(671–680)	ELVNQIIEQL (E10L)	0	7	87	25	[21]
	(956–964)	LLWKGEGAV (L9V)	89	100	93	97	[25]
ENV	Gp120 (120–128)	KLTPLCVTL (K9L)	82	86	96	81	[19]
	Gp120 (120–128)	KLTPLCVSL (K9L-8S)	0	4	0	2	a)
	Gp41 (260–268)	RLRDLLLIV (R9V)	0	65	4	25	d)
NEF	(134–143)	PLTFGWCFKL (P10L)	34	73	100	65	[20]
	(188–196)	AFHHVAREL (A9L)	0	17	15	13	[22]

a) Numbering is based on the amino acid sequence of the HIV 1 WEAU clone 1.60 (Genbank accession no. U21135). Note that the WEAU sequence may not be always identical to that of the reactive peptide and simply indicates its location in the viral proteins.

b) Frequencies were calculated from the NIH website (http://hiv-web.lanl.gov/cgi-bin/EPILIGN/EPI.cgi).

c) Potential epitopes with high affinity for HLA-A0201 molecules.

d) V. Barnaba, personal communication.

The results, given in Table 3, indicate that with only few exceptions (E11Q, R9V) the P1-substituted peptides displayed enhanced affinity and stabilizing capacity for HLA-A0201.

2.4 CTL responses of HHD mice injected with P1 tyrosine-substituted peptides

Mice were injected s.c. with the substituted peptides. Following *in vitro* restimulation with the same peptides, spleen effectors were assayed against ^{51}Cr-labeled HHD-transfected RMA-S cells pre-incubated with either P1 tyrosine-substituted, wild-type or control (Inf.m.G9L) peptides.

Improvement in the cytolytic responses were generally observed in terms of number of responder mice and strength of their responses (Table 4). Notably, CTL responses were induced against Y/P9L and Y/P10L peptides, which were non-immunogenic as wild-type peptides. The CTL responses raised by the substituted peptides were, in almost all cases, able to recognize target cells loaded with the corresponding wild-type peptides. Levels of cross-recognition (ratios of maximal lysis obtained testing target cells sensitized with wild-type versus tyrosine-substituted peptides) ranged between 25 and 105%. Higher levels of cross-recognition were observed when the sensitizing wild-type peptide was further included in the cytolytic assay medium (i.e. P10L, data not shown) a likely reflection of a poor stabilizing capacity.

Table 2. Cytolytic response of HHD mice injected with HIV I-derived CD8 epitopic peptides

CD8 epitopic peptides		Responder/tested mice[a]	Maximal specific lysis (%)[b]
GAG	S9L	4/5	86, 60, 46, 26
	T9V	2/6	22, 19
	E9V	0/6	
POL	L10V	1/6	48
	A9M	3/6	67, 15, 14
	V11V	3/6	57, 18, 16
	V9L	1/6	34
	I9V	4/6	52, 35, 29, 16
	P9L	0/6	
	E11Q	0/6	
	E10L	0/6	
	L9V	3/6	52, 22, 13
ENV	K9L	0/6	
	K9L-8S	3/6	18, 13, 10
	R9V	1/6	14
NEF	P10L	0/6	
	A9L	0/6	

a) Mice were injected s.c. at the base of the tail with 100 µg peptide emulsified in IFA. Seven days later, splenocytes were restimulated for 5 days *in vitro* with syngeneic LPS-lymphoblasts pulsed with the immunizing peptide and tested in a ^{51}Cr-release assay against either relevant or control (inf.m. G9L) peptide-pulsed HHD-transfected RMA-S cells.
b) Maximal specific lysis observed usually at a 100:1 E/T ratio.

One exception was the V11V peptide, which in its tyrosine-substituted form was unable to induce cytolytic responses. Based on these results either wild-type or tyrosine-substituted peptides were selected for further immunizations. Wild-type peptides were chosen if they were of equal or better immunogenicity than their tyrosine-substituted homologues.

2.5 Cross-recognition of the main HIV 1 variants

Whereas some epitopic peptides tested are highly conserved among HIV 1 isolates (E9V, L10V, V11V, V9L, L9V,

Table 3. Relative affinity and stabilizing capacity for HLA-A0201 molecules of wild-type and P1 tyrosine substituted HIV I-derived CD8 epitopic peptides[a]

CD8 epitopic peptides		Wild-type peptides RA[b]/DC[c]	P1 tyrosine substituted peptides RA/DC
GAG	S9L	5.5 / > 6 h	5 / > 6 h
	T9V	5.5 / > 3.5 h	5.5 / > 6 h
	E9V	21 / < 2 h	1.75 / > 6 h
POL	L10V	10 / < 2 h	1.5/4 h
	A9M	5/2 h	1.75 / > 6 h
	V11V	4.5/2 h	2.5/3.5 h
	V9L	> 100/NM	15 / < 2 h
	I9V	1/5 h	NT
	P9L	> 100/NM	15 / < 2 h
	E11Q	> 100/NM	> 100/NM
	E10L	> 100/NM	10 / < 2 h
	L9V	7.5 / > 6 h	5 / > 6 h
ENV	K9L	0.65 / > 6 h	0.4 / > 6 h
	K9L-8S	1.35 / > 6 h	0.7 / > 6 h
	R9V	> 100/NM	> 100/NM
NEF	P10L	> 100/NM	5/NM
	A9L	> 100/NM	7 / > 6 h

a) TAP-, HLA-A0201+ T2 cells were overnight incubated with various concentrations of tested and reference (I9V) peptides.
b) Relative affinity (RA) is the ratio of the concentrations of tested versus reference peptides needed to reach 20 % of the maximal amount of stabilized molecules as defined with high concentrations of reference peptide.
c) Half-life of stabilized HLA-A0201/peptide complexes (DC 50) was evaluated following T2 cells and peptide (100 µg/ml) overnight incubation by measuring at time intervals (0, 2, 4, 6, 8 h) the amount of residual cell surface peptide-HLA-A0201 complexes following peptide removal, T2 cells being maintained at 37 °C in the presence of brefeldine A to block cell surface export of neo-synthetized HLA-A0201 molecules. In all these experiments, the amount of stable peptide-HLA-A0201 complexes was evaluated by indirect immuno-fluoresence and FACS analysis.
NT, not tested, NM not measurable due to too low peptide affinity for HLA-A0201 molecules and in P10L case, peptide toxicity for T2 cells. Whereas RA and DC values were satisfactorily reproducible (variations between experiments in a 10 % range), they underestimate for some peptide their affinity for the HLA-A0201 molecules due to poor solubility or to the presence of a cysteine residue promoting peptide dimerization. In a more extensive study roughly 10 % of the poor binder/poor stabilizer peptides turned out to be good immunogens (K. Kosmatopoulos, unpublished data).

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Table 4. Cytolytic response of HHD mice injected with P1 tyrosine substituted peptides and cross-recognition of wild-type HIV I-derived epitopic peptides

Y/P1 substituted epitopic peptides		Responder/tested mice[a]	Maximal lysis[b]	Cross-recognition of w.t. peptide (% of maximal lysis)[c]	
GAG	Y/S9L	4/6	55, 54, 34, 31	4/4	85%
	Y/T9V	3/5	87, 62, 26	3/3	85%
	Y/E9V	1/6	47	1/1	53%
POL	Y/L10V	0/6			
	Y/A9M	2/6	40, 30	1/2	33%
	Y/V11V	0/6			
	Y/V9L	4/6	21, 17, 17, 16	4/4	105%
	Y/I9V	NT			
	Y/P9L	3/6	96, 63, 55, 11	4/4	53%
	Y/E11Q	0/6			
	Y/E10L	0/6			
	Y/L9V	1/6	30	1/1	43%
ENV	Y/K9L	3/6	28, 27, 23	3/3	76%
	Y/K9L-8S	5/6	73, 60, 56, 33, 23	5/5	80%
	Y/R9V	1/6	64	1/1	25%
NEF	Y/P10L	4/6	90, 75, 37, 25	4/4	48%
	Y/A9L	0/6			

a) Mice were injected s.c. at the base of the tail with 100 µg of tyrosine-substituted peptide emulsified in IFA. Seven days later, splenocytes were restimulated for 5 days in vitro with syngeneic LPS-induced lymphoblasts pulsed with the immunizing peptide and tested in a ^{51}Cr-release assay against either tyrosine-substituted or control (Inf.m.G9L) peptide-pulsed HHD-transfected RMA-S cells.

b) Maximal specific lysis usually observed at a 100:1 E:T ratio.

c) Cross-recognition of wild-type peptide-pulsed HHD-transfected RMA-S cells was tested for each responder mouse. The number of mice cross-recognizing the wild-type peptide versus the number of mice recognizing the corresponding tyrosine-substituted immunizing peptide are given. The levels of cross-lysis, expressed in % correspond to the ratio of the sum of maximal lysis observed with the responder mice tested against wild-type peptide pulsed target cells divided by the sum of maximal lysis obtained when tested against target cells pulsed with the corresponding tyrosine-substituted immunizing peptide. NT, not tested.

P9L) some others (R9V, S9L, T9V, A9M, K9L, P10L) are less conserved and (with the exception of R9V) coexist with variants of significant frequency [31]. Therefore we tested for the latter five epitopes whether the CTL responses elicited by the selected wild-type or tyrosine-substituted peptides cross-recognized their most common variants.

Cross-recognition was generally observed (Fig. 1). Following vaccination with A9M and restimulation with A9M, the CTL recognized A9M-3T, A9M-3T7K and A9M-

3T4A7D variants with the same efficiency as A9M. The P10L variants were cross-recognized as efficiently (P10L-3C, P10L-4L) as or slightly less efficiently (P10L-8Y) than P10L following immunization with Y/P10L. Vaccination with S9L and Y/T9V generated CTL, which, however, recognized the variants S9L-6V, S9L-3F and T9V-9I less efficiently, respectively. Part of these observations may reflect the participation of the substituted residues in the antigenic motif seen by the TCR as best exemplified by the K9L-8S epitopic peptide, which was never recognized following Y/K9L vaccination.
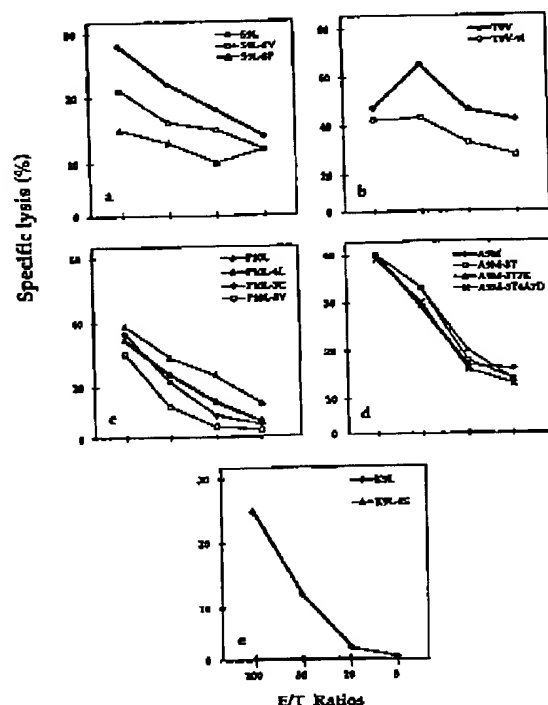
Fig. 1. Cross-recognition of HIV 1 most common variants. Mice were immunized and splenocytes were restimulated *in vitro* with S9L, Y/T9V, Y/P10L, A9M or Y/K9L synthetic peptides as indicated in the legend of Table 2. Cytolytic activity was evaluated against peptide-loaded HHD-transfected RMA-S cells using Inf.m.G9L as negative control. Sequence and frequency among HIV 1 isolates (all clades) of the epitopic variants: S9L (32%), S9L-3F (P3, Y/F, 22%), S9L-8V (P8, T/V, 6%), T9V (39%), T9V-9I (P9, V/I, 34%), P10L (65%), P10L-3C (P3, T/C, 14%), P10L-8Y (P8, F/Y, 6%), P10L-4L (P4, F/L, 5%), A9M (21%), A9M-3T (P3, V/T, 18%), A9M-3T7K (P3, V/T; P7, T/K, 6%), A9M-3T4A7D (P3, V/T; P4, E/A; P7, T/D, 5.5%), K9L (81%) K9L-8S (P8, T/S, 2%).

2.6 DNA polyepitope immunization

A polyepitope containing 13 epitopes in either wild-type (S9L, L10V, A9M, V11V, L9V) or tyrosine-substituted forms (Y/T9V, Y/E9V, Y/V9L, Y/I9V, Y/P9L, Y/K9L, Y/K9L-8S, Y/P10L), without amino acid spacers between epitopes, was introduced into the pre-S2 segment of the middle S hepatitis B protein. HHD mice were injected i.m. with pCMV-B10 recombinant DNA and were individually assayed 3 weeks later. Splenocytes were restimulated *in vitro* in separated cultures twice with each of the 13 epitopic peptides. CTL activity was evaluated using peptide-sensitized HHD-transfected RMA-S target cells.

The results of a representative experiment are illustrated in Fig. 2. With the exception of L9V, all epitopes included in the polyepitope induced specific CTL responses with significant recognition of the wild-type peptides. Globally, the same hierarchy in terms of immunogenicity was observed following immunizations with both polyepitope DNA and purified epitopic peptides in IFA.

To obtain more accurate information on the CTL responses induced *in vivo* following polyepitope DNA immunization, CTL frequencies for each of the 13 wild-type epitopic peptides were evaluated in an IFN-γ ELISPOT assay testing mice individually. The results given in Table 5 confirmed that multiepitopic CTL responses were induced *in vivo* in each immunized mouse. In spite of inter-individual variations in the strength of the responses, a hierarchy of immunogenicity of the epitopic peptides, comparable to that observed following more

Table 5. Frequencies of epitopic peptide-specific IFN-γ-secreting splenocytes HHD mice immunized with DNA polyepitope[a]

CD8 epitopic peptides	IFN-γ ELISPOT numbers per 10^6 splenocytes			
	Mouse 1	Mouse 2	Mouse 3	Mouse 4
S9L	497	170	232	203
T9V	122	33	31	69
E9V	85	47	68	52
L10V	51	32	0	86
A9M	91	29	0	13
V11V	340	30	39	162
V9L	37	51	20	62
I9V	41	26	13	123
P9L	0	29	0	16
L9V	0	59	0	48
K9L	177	305	182	146
K9L-8S	57	47	0	53
P10L	72	48	0	69

a) Splenocytes from HHD mice immunized 21 days earlier with HIV 1-derived DNA polyepitope were cultured in the presence of each 13 HLA-A0201-restricted HIV 1-derived peptides (1 mg/ml, 1 × 10^5 cells/well, 40 h). IFN-γ-releasing cells were then evaluated in an anti-IFN-γ ELISPOT assay. Peptide-specific spot numbers were obtained by subtracting the number of background spots (as determined in wells with Inf.m.58 irrelevant peptide) from the number of spots with HLA-A0201-restricted HIV 1-derived peptide.

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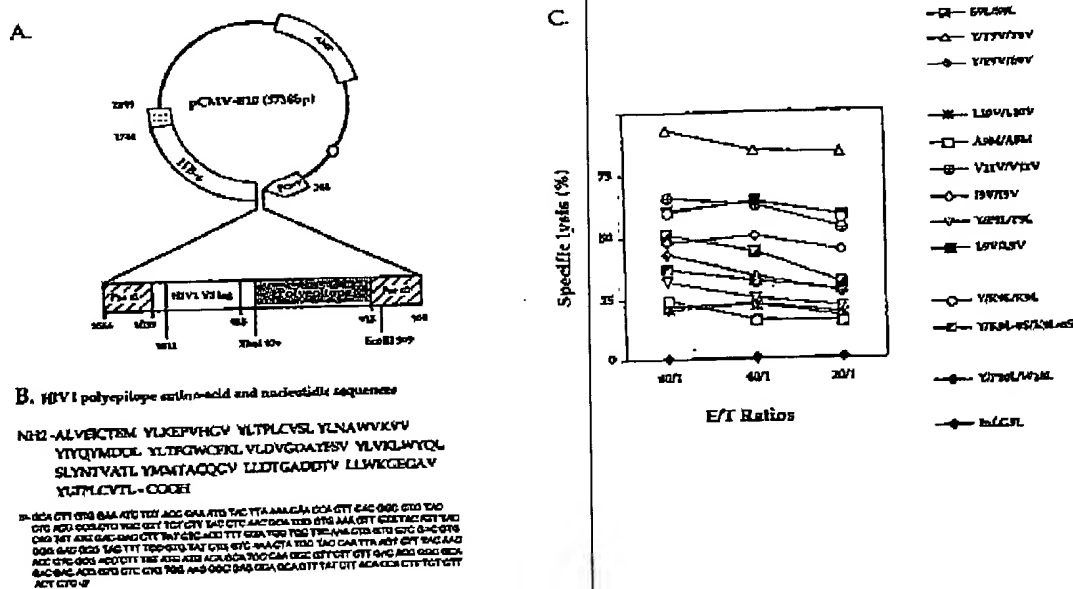
Fig. 2. Polyepitope DNA immunization. (A) Schematic representation of the pCMV.B10 recombinant expression vector. The pCMV.B10 vector is a pcDNA3 derivative (Invitrogen, Costa Mesa, CA), in which the nucleotide sequences of the hepatitis B middle [initiation at Position 900 (ATG), termination at Pos. 1744(TAA)] and small [initiation at Pos. 1066 (ATG), termination at Pos. 1744 (TAA)] envelop proteins have been inserted downstream of a human CMV immediate early promotor. A polyadenylation signal is provided by the HBV untranslated sequence (nucleotides 1744–2899). The central part of the coding pre-S2 segment sequence was replaced by a polylinker in which the HIV 1-derived polyepitope DNA was inserted. An HIV 1-derived (MN isolate) V3 loop tag was inserted, downstream of the polyepitope. (B) Amino acid and nucleotide sequences of the polyepitope. P1 tyrosine-substituted residues are shown in bold characters. (C) CTL response of HHD mice to polyepitope DNA immunization. Mice were injected i.m. with pCMV.B10 recombinant DNA encoding the HIV 1-derived polyepitope. Splenocytes from individual mice were restimulated twice in vitro, 21 days later, for 5 days with synthetic peptides analogous to those included in the polyepitope. Cytolytic assays were performed using HHD-transfected RMA-S cells loaded with corresponding synthetic peptides in their wild-type configuration using Inf.m.G9L as negative control.

prolonged in vitro restimulation and ^{51}Cr-release assay, was observed.

A subsequent and independent experiment was performed to determine whether long-lasting multiepitopic CTL responses could be elicited by the polyepitopic immunization and could recognize target cells presenting naturally processed epitopic peptides. Six mice were individually tested 6 months after immunization. Splenocytes were restimulated in vitro as in the previous experiment and tested against both HHD-transfected RMA-S cells loaded with wild-type peptides (Fig. 3A) or HHD-transfected HeLa cells infected with recombinant vaccinia viruses coding for HIV 1 (LAI isolate), GAG, POL, ENV or NEF (Fig. 3B). The results of a representative mouse (Fig. 3) indicate that long-lasting CTL responses against most of the epitopes included in the polyepitope

could be induced in individual animal. In this latter experiment a L9V-specific response was induced, whereas a A9M response was not, a likely reflection of individual differences in T cell repertoire mobilization. Of peculiar interest, most of the elicited CTL lysed HeLa cells infected with the HIV 1 recombinant vaccinia viruses. One noticeable exception, the Y/P10L' CTL responses failed to lyse HeLa cells infected with HIV 1 NEF (LAI-isolate) recombinant vaccinia viruses, a possible consequence of the expression by this isolate of the P10L-8Y epitopic peptide variant less efficiently cross-recognized (Fig. 1c).

Therefore, in vivo immunization with the HIV 1 polyepitope construct elicited multiple CTL responses in an individual mouse of sufficient strength for recognition of cells presenting naturally processed HIV 1 antigens.

Specific lysis (%)
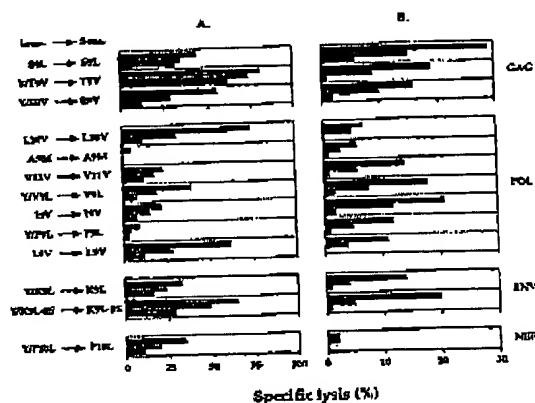
Fig. 3. Recognition of naturally processed antigens (see also legend to Fig. 2). Polyepitope DNA-immunized splenocytes from a single mouse were restimulated *in vitro* with each immunizing (Imm.) peptide, and assayed against wild-type HIV 1-derived or control (Inf.m. G9L) peptide-sensitized (sens.) HHD-transfected RMA-S cells (A) and HHD-transfected HeLa cells infected with either GAG, POL, ENV or NEF HIV 1 recombinant (LAI isolate) vaccinia viruses (B) according to the viral protein origin of the peptides. Specific lysis at 50, 12.5 and 3 E/T ratios (from top to bottom) were calculated by subtracting the nonspecific lysis obtained with control (Inf.m.G9L) pulsed or wild-type vaccinia virus infected targets.

3 Discussion

Unlike acute viral infections, often characterized by oligoepitopic immunodominant CTL responses [32], the long-lasting antigenic challenge of an HIV infection is often associated during the latent chronic phase of the disease with the development of polyepitopic CTL responses in infected individuals. Whereas such polyepitopic responses during the primary infection have been associated with a better prognosis [9, 10], their emergence is usually delayed, reflecting asynchronicity in the development of different responses and variations in their capacity to persist over time. It is likely that such variability reflects in part intrinsic differences in the immunogenic potential of the different epitopes.

A significant number of the HIV 1-derived native epitopes tested in this study failed to induce significant CTL responses, and these failures correlate quite satisfactorily with a low affinity for HLA-A0201 molecules and a low stabilizing capacity of these epitopic peptides. To be of interest for vaccine development, their immunogenicity has to be implemented in the respect of their antigenicity. The P1 tyrosine substitution has, in most cases,

fulfilled this goal. Such an enhancing effect was first documented for the I9V epitopic peptide with human CTL [30]. A similar observation was made with a melanoma epitopic peptide (Melan A/MART1.27) where the tyrosine residue was added at its N terminus [33]. More recently, we generalized these observations to a series of 30 human tumor-derived epitopic peptides [28]. In the present study, this enhancing effect was documented for seven additional, HIV 1-derived, epitopic peptides and we could document cross-recognition of the corresponding wild-type epitopic peptides. The possibility to induce CTL responses specific for multiple epitopes, including responses against normally weak even cryptic epitopes may not only be beneficial in preventive vaccination but could also be of immunotherapeutical interest in HIV 1 infected patients. Indeed, these weak or cryptic epitopes may not have been immunologically driven to mutate and the corresponding CTL clones not downregulated or deleted as has been observed in certain cases [34].

The tyrosine enhancement of affinity and stabilizing capacity could be due to the development of a stacking interaction with the aromatic ring of tryptophan 167 in HLA-A0201, as crystographically documented for the hepatitis B nucleocapsid 18–27 epitopic peptide which has a natural P1 phenylalanine [35]. With few exceptions (A9M, V11V), tyrosine substitution did not alter the antigenicity for CTL of the HIV 1 peptides. The terminal position of the P1 residue side chain in the antigenic surface presented to the TCR [35], and the immediate vicinity of the main anchor residue (Leu in P2 for HLA-A0201-bound peptides), probably conceal the structural modification to a limited area only scanned by few CTL. The two documented exceptions, however, justify a case by case verification of the expected immunological beneficient of such substitution [36]. The tryptophan 167 being conserved among most HLA class I alleles, the P1 tyrosine effect might apply to other HLA class I molecules. However, the structural polymorphism of HLA class I molecules, including that of their anchor positions, makes it likely that the antigenicity of tyrosine-substituted peptides will not be always as well preserved as in the case of HLA-A0201.

The transposition to humans of immunological observations made in mice, of which the immune system has been partially humanized, is still a matter of debate. Tolerance to species-specific self peptides, functional differences in the antigen-processing machinery of the two species, as documented for the TAP-peptide pumps [37], should modulate differentially to some extend the capacity of the two species to mount CTL responses. However, evidences reported in the literature [16, 38, 39] as well as those that we have accumulated in our labora-

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tories (immunodominance in the two species of the CTL response against the same influenza matrix peptide [17], validation in humans of new epitopic peptides from a human oncogene following their identification in HHD mice; K. Kosmatopoulos et al., unpublished results) support the notion of a substantial overlap. For studies where humanized mice are used to improve the immunogenicity of epitopic peptides already described in humans, the concern of non-transposition is rather limited. For studies, using the same mice, aiming at the identification of new epitopic peptides, a validation in human is required, but will be simplified by focussing on the most promising peptide candidates selected with the humanized animals.

To be of real value, an HIV vaccine should not only simultaneously induce CTL responses against several epitopic peptides, but also target conserved peptide motifs or induce responses recognizing most of the variants. These two goals are satisfactorily reached with the polyepitopic construct described. With the exception of the K9L and K9L-8S (81% and 2%, respectively, of the HIV isolates), for which the lack of cross-recognition led us to introduce both motifs in the polyepitope, for all other epitopes, the sequences were highly conserved among isolates or the induced responses cross-recognized the main variants. Despite the extensive structural variability of HIV, a polyepitope construct of potential vaccine interest can be devised for HLA-A0201 individuals. DNA immunization was well adapted for mouse experimental purposes. Its efficiency in primates and feasibility on a mass scale in man are, however, still matters of speculation and debate. The HHD mice are certainly well adapted to evaluate other vaccine strategies as exemplified in a parallel study with a recombinant vaccine [40].

4 Materials and methods

4.1 Mice

HHD mice express a transgenic monochain histocompatibility class I molecule in which the C terminus of the human β2m is covalently linked to the N terminus of a chimeric heavy chain (HLA-A0201 α1-α2, H-2D^b α3 -transmembrane, and intracytoplasmic domains). The H-2D^b and mouse β2m genes of these mice have been disrupted by homologous recombination [17]. All mice used were bred in our animal facility.

4.2 HLA-A0201 peptide binding and stabilization assays and immunization procedures

Peptides, purchased from either NEOSYSTEM (Strasbourg, France) or SYNTEM (Nimes, France), were dissolved in dimethylsulfoxide (DMSO, 20 μl/mg peptide) then diluted in PBS (2 mg/ml). Peptide binding and stabilization assays were performed as already described [28]. Mice were injected s.c. at the base of the tail with 100 μg of a HLA-A0201-restricted peptide, with or without 140 μg of the helper peptide (HBV core 128–140, TPPAYRPPNAPIL, HBV core T13L), emulsified in IFA (DIFCO, Detroit, MI) 7 days before in vitro restimulation.

4.3 In vitro restimulation of effector cells, cytolytic and IFN-γ enzyme-linked immunospot (ELISPOT) assays

Spleen cells from primed mice were restimulated using irradiated, peptide-loaded (5×10^6 cells/ml, 10 μg/ml peptide, 2 h at RT in FCS-free RPMI medium), LPS-induced HHD lymphoblasts. On day 6, cultured cells were tested in a 4 h ^51Cr-release assay, using as targets either HHD-transfected TAP⁻ RMA-S cells loaded with relevant or negative control Influenza matrix 58–66 (GILGFVFTL Inf.m.G9L) peptides (10 μg/ml) or HHD-transfected HeLa cells infected overnight (10 PFU/cell) with either recombinant (HIV 1 LAI isolate) GAG, POL, ENV or NEF or control wild-type (Copenhagen strain) vaccinia viruses. Specific lysis was calculated as follows: (experimental release – spontaneous release)/(total release – spontaneous release)×100. The number of epitopic peptide-specific IFN-γ-secreting splenocytes was determined by ELISPOT as already described [41].

4.4 Polyepitope recombinant HBs DNA construct

The polyepitope DNA was constructed, using partially complementary oligonucleotides (Genset, Paris, France) and PCR amplification. The final construct was inserted between the EcoRI and XhoI sites of the pCMV-B10 expression vector. Recombinant plasmids were purified on LPS-free QIAGEN columns (QIAGEN, Hilden, Germany). Mice were injected i.m. with 10 μM cardiotoxine (LATOXAN, Rosans, France) in 50 μl PBS and, 5 days later, with 100 μg pCMV-B10 DNA for a 21-day priming.

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Review

Polytope vaccines for the codelivery of multiple CD8 T-cell epitopes

Andreas Suhrbier

Vaccines against a number of diseases, including HIV, Epstein Barr virus, malaria and several cancers, are believed to require the coinduction of multiple αβ CD8+ cytotoxic T-lymphocyte responses that are directed towards a number of different target antigens. The difficulties associated with making large recombinant vaccines that contain numerous antigens has led to the development of αβ CD8+ cytotoxic T-lymphocyte polyepitope or polytope vaccine approach, where multiple (usually 8–10 amino acids long) αβ CD8+ cytotoxic T-lymphocyte epitopes, derived from several antigens are conjoined into single artificial constructs. Such polytope constructs can be delivered using a number of different vaccine vector modalities with each epitope in the construct emerging as individually immunogenic.

αβ CD8+ cytotoxic T-lymphocytes (CD8 T-cells) have emerged as crucial components of protective immune responses directed against a number of diseases, including HIV, Epstein Barr virus (EBV) and other Herpes viruses, several cancers and the liver stage of malaria. Unfortunately, the short (usually 8–10 amino acids long) sequences or epitopes that are recognized by CD8 T-cells in association with products of the class 1 major histocompatibility complex (MHC) occur relatively rarely within protein sequences. Thus, an individual may only generate CD8 T-cell responses specific for one or two (often zero) epitopes within a given foreign protein [1]. The situation is further complicated in the case of HIV, where an individual may be challenged with, or develop during infection, HIV variants with epitope mutations that evade the CD8 T-cell responses directed towards the original epitope [2–5]. Clearly, if an immune response contains CD8 T-cell responses directed at multiple epitopes, the probability that the HIV virus can mutate all the targeted epitopes and completely escape immune control is greatly reduced. Most tumors also present a unique problem, since the expression of cancer antigens, which can be targeted by anticancer

CD8 T-cells, is often highly variable even within the same tumor mass [6,7]. Individual tumor antigens and MHC alleles can also be lost during development of the cancer. Thus, CD8 T-cell responses directed at multiple cancer antigens and restricted through several MHC alleles promise to be more effective, since a greater percentage of the tumor cells should become subject to immune attack [8–10]. The targets of protective CD8 T-cell responses against EBV include nuclear and latent antigens, which have been shown to have oncogenic potential.

The development of prophylactic EBV vaccines against infectious mononucleosis (IM) and post-transplant lymphoproliferative disease (PTLD) have therefore favored the use of epitope-based vaccines. To be able to cover a human population that has a substantial diversity of human leukocyte antigen (HLA) alleles, epitopes restricted by a number of common HLA and derived from several EBV antigens need to be combined [11–13]. An epitope-based approach has also been favored for human papilloma virus (HPV) vaccines to avoid using the oncogenic E6/E7 proteins [14]. For vaccines directed at the liver stage of malaria, the desire to target multiple liver

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Table 1. Polytope and related vaccines in murine systems.

Disease	Vector	Flanking sequences	No. epitopes and restriction	Ref.
LCMV	Vaccinia	Yes + Kozac and initiation Methionine	2 H-2^{b,d}	[63]
Epstein Barr virus	Vaccinia	None	9 Various HLA	[13]
Various	Vaccinia	None	10 H-2^{d,k,b}	[19]
Various viruses	Vaccinia	None (+B & CD4 epitopes)	3 H-2^{d,b}	[64]
HIV	Vaccinia	None	10 HLA-A2	[2]
Melanoma	Vaccinia	None	10 HLA A2	[9]
Various	DNA	None	10 H-2^{d,k,b}	[41]
Papillomavirus	DNA	Yes – AAY- +B + CD4 epitopes	6 HLA A2/24, H-2D^{b}	[18]
HIV + Hepatitis B	DNA	None	9 HLA A2/11	[17]
HIV	DNA	None	20 HLA A2/11, B7	[16]
HBV		None and 1	9 HLA A2/11	
HIV	Hepatitis B fusion DNA vaccine	None	13 HLA with altered ligands	[33,65]
Melanoma			10 HLA A2	
HIV	DNA/modified vaccinia Ankara	None	2 H-2d, 4HLA	[3,5]
Malaria	DNA/modified vaccinia Ankara	None	1 H2Kd plus *Plasmodium falciparum* epitopes	[66]
Influenza	Lipopeptides	Yes	3 H-2^{d,k,b}	[67]
Hepatitis C	Lipidated peptides	AAA-	2 HLA	[68]
Various	*B. pertusis* adenylate cyclase	None	2 H-2^{d}	[69]
			1 H-2Kb	
Tumors	Recombinant adenovirus	AAA- spacers	4 H-2^{b}	[8]
			2 HLA A2	
Melanoma	Adenovirus + dendritic cells	None	8 HLA A2, A1	[30]
Papillomavirus	Chimeric papillomavirus-like particles	+ B and CD4	3 H-2^{b}, H2^{d}, HLA A2	[14]
Malaria	Ty particles	None	15 mouse and human	[15]
Various	ISCOM	None	4 H-2^{d}	[22]
Various	Kunjin replicon	None	10 H-2^{d,k,b}	[27]

A: Alanine; *B. pertusis*: *Bordetella pertusis*; HBV: Hepatitis B virus; HLA: Human leukocyte antigen; ISCOM: Immunostimulating complex; LCMV: Lymphocytic choriomeningitis virus; Y: Tyrosine.

stage antigens and cover several HLA alleles has also led to the adoption of the multiepitope approach [15]. These often disease-specific reasons, combined with the difficulty of constructing vaccines containing large numbers of recombinant proteins, have led to the adoption of the polyepitope or polytope vaccine approach for delivering multiple CD8 T-cell epitopes. The polytope approach simply combines multiple CD8 T-cell epitopes end to end to produce an artificial construct, which can be delivered by a number of different vaccine modalities. Perhaps surprisingly, every individual epitope within such conjoined strings of epitopes has been shown repeatedly to be immunogenic (TABLE 1).

Polytope vaccine research & development: an overview

An increasing number of polytope vaccines against several diseases and delivered by a number of different vaccine vector systems have now been shown to induce multiple protective CD8 T-cell responses in several murine and *in vitro* systems (TABLE 1). Unfortunately, only a limited number of vector systems have so far shown safe and efficient induction of CD8 T-cell responses in humans.

Nevertheless, several polytope-based vaccines are the subject of commercial development, have been tested in monkeys and/or are near or in Phase I human clinical trials using vector systems that have shown promise in humans (TABLE 2).

Polytope™ is a trademark of Vaccine Solutions Pty Ltd., Queensland, Australia, who holds a generic patent application on polytope technology [201]. This patent has been licensed to CSL Ltd., Victoria, Australia and Bavarian Nordic, Copenhagen, Denmark. Oxxon Pharmaccines Ltd, Oxford, UK, holds patent applications on their HIV construct [202] and prime boost strategy [203]. Another patent has also been filed in the polytope area [204].

Interepitope spacers & epitope order

Most of the polytope constructs tested so far have conjoined minimal epitopes without any intervening sequences and the majority of epitopes tested have so far been shown to be immunogenic (TABLE 1 & 2). Recently, several reports have suggested that the immunogenicity of individual epitopes might be enhanced by adding appropriate amino acids between the minimal epitopes to improve their proteolytic processing and therefore immunogenicity [16–18]. In contrast, other studies have shown that flanking amino acids other than proline (P) and glutamine (G), which are rarely found at the end of epitopes – see discussion [19] – have minor influences on immune induction and that epitope immunogenicity is primarily regulated by residues within the epitope [20–22]. Experiments seeking to test the role of spacers may often be difficult to interpret, since changing the nucleotide sequence of these artificial gene constructs can have unforeseen influences on transcription, mRNA stability and translation efficiency. The protein sequence can also influence polypeptide stability, secondary structure and

physicochemical characteristics [23,24]. Whether polytope vaccines can be universally improved with specific interepitope spacers remains an open question. Current data would suggest that most polytope vaccines efficiently induce protective CD8 T-cell responses without spacers, although individual epitopes may benefit from added flanking regions in specific cases.

The order of epitopes within a polytope have also been shown in some [17,25], but not other studies [22], to influence the immunogenicity of certain epitopes. Similar arguments to those given above apply and unfortunately until rules (if any) emerge, empirical testing of all possible combinations for even a small 10 epitope polytope would, for example, require the testing of over 3 million different constructs.

Immunodominance & immunodomination

It is well established that a hierarchy of CD8 T-cell responses from subdominant to dominant can be seen in many diseases and following polytope vaccination [22,26,27]. However, the question of whether dominant responses effectively suppress or interfere with induction of less dominant responses (immunodomination) remains controversial and potentially poses a problem for polytope vaccines [26,28,29]. In our hands, the removal of a dominant HLA A2 epitope from an immunostimulating complex (ISCOM)-based polytope vaccine being tested in transgenic mice did not improve responses to subdominant HLA A2 epitopes within the same vaccine (unpublished data). Furthermore, pre-existing CD8 T-cell responses to one epitope in a recombinant vaccinia polytope vaccine did not inhibit the induction of responses to the other epitopes in that vaccine in mouse models, unless the pre-existing CD8 T-cell responses were very substantial [28]. In contrast, Rodriguez et al., have illustrated strong IFN-γ-mediated immunodomination effects in minigene

Table 2. Some polytope vaccines in, or near to, human clinical trials.

Disease target	Construct	Vector modality	Company/organisation
Malaria liver stage	*Plasmodium falciparum* epitopes linked to thrombopsondin-related adhesion protein	DNA/modified vaccinia Ankara	Oxxon Pharmaccines Ltd, Oxford, UK [15,52]
HIV	25 CD8 T-cell epitopes linked to HIV-1 gag	DNA/modified vaccinia Ankara	Oxxon Pharmaccines Ltd, Oxford, UK [4,5]
EBV, glandular fever, PTLD	26 CD8 T-cell epitopes from EBV antigens fused to a fragment of gp350	Recombinant protein + ISCOM	CSL Ltd, Victoria, Australia
Malignant melanoma	8 HLA A2/1 melanoma epitopes	Adenotransferred dendritic cells	ICRF, London, UK [30]
Advanced breast cancer	12 HLA A2 epitopes, HER2/neu [70]	As above	ICRF, London, UK§
HIV	Multiple epitopes, collaboration with Epimmune Inc., San Diego, CA, USA	Modified vaccinia Ankara	Bavarian Nordic, Coppenhagen, Denmark

EBV: Epstein Barr virus; HLA: Human leukocyte antigen; ICRF: Imperial cancer research fund; ISCOM: Immunostimulating complex PTLD: Post-transplanatation lymphoproliferative disorder. §Scardino et al. An oligoepitope DNA vaccine targeted to HER2/neu expressing tumors (submitted).

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DNA vaccines and suggests that separation of the epitopes can overcome the problem [29]. Smith *et al.* also showed *in vitro* immunodomination effects after repeated restimulation with dendritic cell (DC)-based polytope vaccines [30]. The science of immunodomination will probably remain an important issue for polytope vaccines, but the magnitude of the effects may be dependent on the vector system used and the epitopes involved [22,31]. Furthermore, effective protection against certain diseases may be adequately mediated by subdominant memory responses that are amplified after challenge [11,22].

Modified MHC-binding epitopes & polytope vaccines

The immunogenicity of subdominant epitopes can be enhanced by changing residues within the epitope that anchor the epitope to its restricting MHC [32]. This approach has been applied to the design of polytope vaccines against HIV [33] and breast cancer, with the latter expected to enter human trials shortly (TABLE 2 Scardino and Kosmatopulos, personal communication). Potential problems with this approach may be the induction of CD8 T-cell responses with subtly altered fine specificity that fail to recognize the parent epitope efficiently [34,35] and/or the more remote possibility of producing antagonist epitopes [36]. Nevertheless, appropriately chosen epitope analogues may find clinical application for stimulating or boosting otherwise subdominant CD8 T-cell responses [37].

CD4 T-cell help

It is well established that CD4 T-cells help in the priming and maintenance of CD8 T-cell responses [38,39]. Specific vaccination modalities like DNA may also provide help independently of CD4 cells [40,41] and virus vectors are clearly going to provide help to polytope epitopes *via* CD4 T-cell responses to the viral vector proteins [42]. However, the question for polytope vaccine designers is often whether this CD4 T-cell help should be specific for the target pathogen (cognate) or if CD4 responses to irrelevant antigens (noncognate) will suffice. Developers of prophylactic EBV, HIV and malaria polytope vaccines have all joined whole proteins or fragments of proteins from the target organism to the polytope constructs to supply a source of CD4 help (and provide antibody responses) (TABLE 2). However, highly immunogenic, unrelated proteins have been successfully used as sources of noncognate CD4 help in a number of epitope-based therapeutic vaccine trials [43–45]. If the vaccine is intended for immunotherapy and the patients cognate CD4 responses are compromised by the disease, noncognate sources of help may be more effective for priming and/or maintenance of vaccine-induced CD8 T-cell responses [2,9,43,44]. Noncognate help might also be supplied by CpG motifs [46], antiCD40 antibodies [47] or antiCTLA4 antibodies [48].

Alternative strategies

A number of other approaches for delivering multiple CD8 T-cell epitopes have been developed and include the construction of multiple antigen peptides [49,50] or the use of a cocktail or library of DNA plasmids encoding a large range of antigens

[51,52]. These approaches may be more difficult to develop into products, as complex and/or multicomponent vaccines are likely to suffer from increased regulatory and quality control problems. Certainly, attempts to make vaccines comprising multiple peptide epitopes in water-in-oil emulsions suffer from a number of problems, including solubility, chemical modifications and oligomerisation [53], although peptide-based immunization does appear to overcome induction of weak responses by subdominant epitopes [22].

Better understanding of the structure–function relationships of potentially hazardous immunogens such as HPV E7 has allowed mutational ablation of transforming activity, which may thus remove the requirement for an epitope-based approach [54].

Conclusions

The value of polytope vaccines in the fight against human disease will have to await the outcome of human clinical trials (TABLE 2). Studies in monkeys have already suggested that polytope vaccines are immunogenic in primates [5,55]. The first promising results of human trials to emerge are those using the malaria polytope (TABLE 2) (A Hill, personal communication). Hand-in-hand with the development of polytope vaccines will be the development of safe vaccine vectors capable of inducing effective CD8 T-cell immunity in humans – many of these vector systems are only now entering Phase I or II clinical trials.

Expert opinion

The polytope approach relies on the identification of CD8 T-cell epitopes, which in turn relies on accurate information about class I HLA alleles. In western countries, the study of HLAs is well advanced due to extensive transplantation programs. The same cannot be said for populations in or from developing countries, where the identity, frequency and/or subtype distribution of many HLAs remains poorly characterized and thus, CD8 T-cell epitope mapping is less advanced. The problem is compounded when the disease is present in multiple ethnic groups, which differ in their HLA profiles. Although the appropriate information is being gathered it may be some time before, for example, a prophylactic HIV polytope vaccine can be designed that will be effective in the majority of the global populations currently at risk from infection [56]. The problem is less acute for an EBV glandular fever vaccine, as this disease is largely restricted to developing countries.

Research to improve the immunogenicity of polytope vaccines may continue to seek universal rules regarding spacers and epitope order. However, equal consideration might be given to improving transcription, stability and translation of mRNA encoding polytope proteins [23,24]. Methods for improving cross-priming might also be considered for vector systems that utilize this route [57]. Any improvements derived from such studies and/or other improvements in vector systems may also influence the relative responses to subdominant and dominant epitopes [22,29].

A rather unhelpful view expressed by some regulators may be that each epitope constitutes an individual vaccine component

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and thus each epitope must first be shown to be individually beneficial in humans before it can be added to a polytope vaccine. Hopefully, such an ill-conceived view will not prevail given that most current vaccines contain components or sequences with no proven benefit and many current vaccines also contain multiple CD8 T-cell, CD4 T-cell and B-cell epitopes that have not been shown to be individually beneficial.

Five-year view
In future clinical evaluations of polytope vaccines, poor performance of the polytope approach may be difficult to distinguish from poor performance of vectors and/or the epitopes. A good performance by a polytope vaccine may equally lead to uncertainty regarding the relative benefit of the polytope approach *versus* the benefits imparted by an individual epitope and/or a codelivered whole antigen or antigen fragment. As it is unlikely that polytope vaccines are going to be extensively tested head-to-head with single epitope or whole-antigen vaccines in human trials, the benefit of the polytope approach in humans may only emerge from extensive correlation between CD8 T-cell response data and clinical outcomes.

As vaccines are being applied to an increasing number of therapeutic applications, polytope vaccines may find application in the development of immunotherapeutics against autoimmune diseases. Self-reactive CD8 T-cells are now implicated in a number of autoimmune diseases [58–60] and

vaccines encoding Fas ligand have been shown to produce antigen-specific T-cell tolerance [61]. One could thus envisage therapeutic antiautoimmunity polytope vaccines codelivered with Fas ligand or interleukin-4 to produce specific CD8 T-cell tolerance.

Key issues

- Will spacers between epitopes improve polytope vaccines?
- How important are immunodomination effects and what is the best method for improving responses to subdominant epitopes?
- When should sources of cognate CD4 help be codelivered with polytope vaccines?
- Are prophylactic polytope vaccines for the developing world and multiple ethnic backgrounds possible?
- Will the benefit or otherwise of the polytope approach be easily distinguishable in human trials from the benefits imparted by individual epitopes, codelivered cognate proteins and/or vector systems?
- Do polytope vaccines have a role in the development of therapeutic anti-autoimmunity vaccines?

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Review

Multi-epitope DNA vaccines

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Summary The evolution of vaccine strategies has seen a move from whole organisms to recombinant proteins, and further towards the ultimate in minimalist vaccinology, the epitope. The epitope-based approach is clearly compelling as only a relatively tiny, but immunologically relevant, sequence is often capable of inducing protective immunity against a large and complex pathogen. The post-reductionist era in epitope-based vaccinology has seen a quest to re-construct complexity and design vaccines containing many epitopes. The hope is that such multi-epitope vaccines might induce immunity against multiple antigenic targets, multiple strain variants, and/or even multiple pathogens. The ability of DNA vaccination to co-deliver a series of antibody and/or CD4 T cell epitopes remains largely unexplored. Successful viral vector and DNA-based experimental vaccines coding for multiple contiguous CD8 CTL epitopes have, however, recently been described. This simple CTL poly-epitope (or polytope) strategy may find application in the design of vaccines against several diseases including EBV, HIV and cancer.

Key words: cytotoxic T lymphocyte, DNA, EBV, epitope, HIV, melanoma, polytope, vaccine.

Vaccines containing multiple antibody epitopes

Vaccines that could generate an antibody response to multiple serotypes would clearly be of some value against pathogens such as *Streptococcus* or influenza where different serotypes cause disease. Synthetic peptide vaccines comprising linear antibody and Th epitopes combined as multiple antigen peptides (MAP) have been known for some time.[1] The original chemistry, however, placed practical limits on the number of different epitopes that could be included in a MAP, although mixing different MAP appears feasible.[2] A recently developed acryloylation-based chemistry now allows large numbers of different epitopes to be co-polymerized into a single homogeneous covalent structure.[3,4] Despite these developments, two fundamental restrictions are imposed on multi-B cell epitope vaccines. First, most antibody epitopes are not linear and are comprised of a complex three-dimensional array of scattered residues[5] and are therefore not readily represented by a linear amino acid sequence. This problem may be partially ameliorated by covalently holding linear sequences in specific conformations,[6] by using linear mimotope mimics of three-dimensional epitopes[7] or by flanking the epitopes with sequences which will fold the epitope into the desired conformation.[8] Second, vaccinees who have had prior exposure to one or more of the epitopes in a multi-B cell epitope vaccine may not respond well to other epitopes in the vaccine because of the 'original antigenic sin' (OAS) phenomenon, which results in the boosting of pre-existing B cell responses at the expense of priming naive B cells specific for the new epitopes.[9,10]

Could multiple linear, appropriately folded or mimotope antibody epitopes be combined in single plasmid DNA constructs? Recently An and Whitton[11] showed that a multi-epitope recombinant vaccinia construct containing a linear antibody epitope could induce an antibody response, suggesting that such an approach might be feasible. In contrast, a malaria antibody epitope delivered in a similar manner did not induce an antibody response.[12] The stability of the artificial multi-epitope protein may be critical,[13] since the multi-epitope protein could be detected when expressed by recombinant vaccinia in mammalian cells in the former but not the latter study. Thomson *et al.*[12,14] suggested that artificial proteins may often be rapidly degraded in mammalian cells because of their lack of secondary or tertiary structure. For a DNA vaccine coding for multiple B cell epitopes to work, the multi-epitope protein would have to: (i) survive long enough to exit the transfected cell in which it was synthesized; (ii) be taken up by surface Ig on a B cell; and (iii) be processed by class II processing enzymes resulting in the liberation of a Th epitope, which can then be presented to Th cells. Whether strategies can be developed which can fulfil these requirements and generate multiple independent B cell responses using DNA vaccines remains to be tested. Ultimately the strategies may not be generic and combining B cell epitopes might be better achieved using different technologies. Alternatively, the epitope-based approach might be avoided altogether and multiple individual DNA plasmids coding for each serologically distinct antigen might simply be co-delivered,[15] a strategy which would generate conformational responses and may also avoid OAS. Recently, Merck reported the testing of a seven-plasmid combination influenza DNA vaccine in monkeys.[16]

Vaccines containing multiple CD4 T cell epitopes

Early experiments showed that endogenous (cytoplasmic or nuclear) expression of CD4 T cell epitopes did not result in efficient loading of class II MHC.[17] Efficient class II presentation was, however, achieved when antigen was targeted to

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Multi-epitope DNA vaccines

endosomal or lysosomal compartments by construction of fusion proteins containing specific signal sequences from the invariant chain.[18] LAMP-1[19,20] or lysosomal enzymes.[21] Nevertheless. An and Whitton[11] showed that at least one of the two Th epitopes in their string of beads construct. which did not contain such signal sequences. could induce a Th response. Coupled class II epitopes have also been delivered as peptide vaccines: however. strong responses against new junctional determinants composed of sequences derived from both epitopes were unexpectedly generated.[22,23] Whether multiple contiguous class II epitope constructs will be able to reliably induce multiple Th responses *in vivo* using DNA vaccination remains to be tested (S Thompson *et al.* unpubl. data, 1997). The relative abundance of CD4 T cell epitopes compared to CD8 epitopes may limit the need for multi-Th ep 　 constructs in the design of vaccines against pathogens. However, such constructs may find application in th 　 　utic vaccines designed to delete, inactivate or deviate the cytokine secretion patterns of specific CD4 T cells responsible for autoimmunity or allergy.[24,25]

Polytope approach for multiple CTL epitope delivery

The peptide epitopes recognized by CD8+ αβ CTL are mainly generated from cytosolic proteins by proteolysis mediated by the multicatalytic proteasome complex.[26,27] The specificity of the proteolytic processing events and the role of LMP2 and LMP7 are reviewed elsewhere:[26,27] essentially proteasomes cleave on the carboxy side of hydrophobic, basic and acidic residues with the specificity for the latter decreasing when interferon-inducible LMP proteins are associated with the proteasome. The influences of sequences flanking CTL epitopes on the proteolytic processing events and the subsequent generation of CTL epitopes remains controversial. Profound i 　 ences of flanking sequences have been reported.[28-33] h 　 ever. these can often, but not always, be explained by th 　 resence of glycine or proline residues adjacent to the n 　 hal epitope. Peptide bonds involving glycine and proline residues, with hydrogen and secondary imide side chains, respectively, are often relatively resistant to protease activity and are also likely to be poorly cleaved by proteasomes.[32] Despite these potential negative influences. CTL epitopes which were moved into novel locations within different proteins were often fully capable of inducing CTL responses.[34,35] Designing minigene vaccines containing multiple CTL epitopes was initiated by Oldstone *et al.*[36] and Whitton *et al.*[37]

who combined two potentially independently initiated minigenes. which coded for sequences containing single CTL epitopes. Single epitopes devoid of flanking sequences. delivered as minigenes by vaccinia or DNA vaccination. have also been shown to be capable of inducing CTL responses.[38,39] Recently we were able to show that each epitope within an artificial polyepitope (or polytope) protein comprising nine minimal CTL epitopes in sequence was processed and presented to appropriate human CTL clones.[14] A similar polytope construct containing 10 contiguous minimal murine CTL epitopes and delivered by recombinant vaccinia, was also able to induce, in mice, MHC-restricted primary CTL responses to all 10 epitopes.[12] In both these polytope constructs the CTL epitopes were devoid of any sequences naturally found to flank the epitopes in their proteins of origin. The latter murine polytope was also successfully delivered using DNA vaccination (S Thompson *et al.* unpubl. data, 1997). Interestingly, the induction of CTL responses by the murine polytope DNA plasmid occurred in the apparent absence of a source of co-expressed CD4 T cell helper epitopes. Although helper independent CTL induction has been reported, the immunostimulatory sequences within plasmid DNA[40] may themselves be sufficient to stimulate APC to prime CTL in the absence of CD4 T cell-derived help.[41]

Several studies have shown that vaccines comprising multiple contiguous minimal CTL epitopes can induce universal priming to each epitope. despite potential interference from processing. competition or immunodominance phenomena (Fig. 1).[11,12,42] The universal priming achieved by these multi-CTL epitope vaccines. argues that the potential negative influences of flanking sequences on processing are likely to be rare or not absolute. Although specific flanking sequences may reduce the amount of epitope generated. the ability to prime a response is likely to represent a threshold phenomenon.[43] Once the threshold is reached, the level of epitope presented to the immune system beyond this threshold may have limited effects on the efficiency of CTL induction.[44,45] Furthermore, the subsequent development of memory CTL. the all important recall response. and the protective activity of the recalled CTL. are all even less likely to be related to the initial level of peptide presented by a polytope vaccine during priming.[46]

Universal priming might not be achieved if subdominant epitopes with poor MHC binding affinity were included in a polytope. however. inclusion of multiple copies of such epitopes in a polytope might overcome this potential problem.

Figure 1 The CTL polytope vaccine concept. An artificial polytope gene coding for multiple contiguous CTL epitopes derived from a series of antigens.

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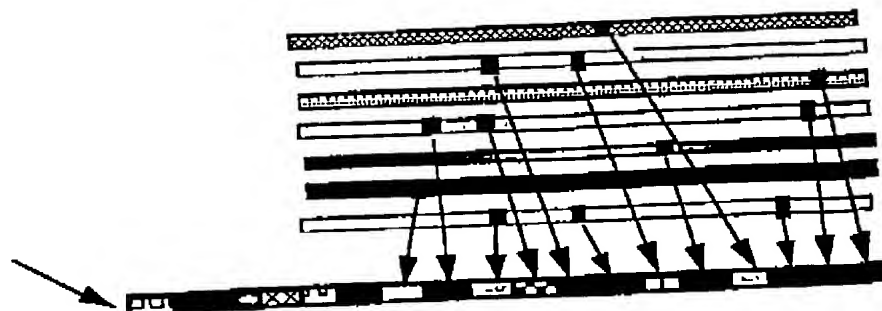
Another consideration which may limit the application of polytope vaccines might be the inability to prime new CTL responses in an individual who already has a CTL response to one of the polytope's epitopes. However, recent experiments suggest that a pre-existing CTL response to one epitope in the murine polytope did not prevent induction of primary CTL responses to the other epitopes in the polytope, indicating that 'CTL OAS' does not appear to present a problem (MA Sherritt *et al.*, pers. comm., 1997).

Several groups have adopted synthetic peptide epitope-based modalities[47,48] to deliver multiple CTL epitopes. The potential advantages of a vectored polytope approach over the use of large peptide mixtures are: (i) peptide formulation, mixture validification, poor solubility, inter-peptide competition and adjuvant issues are avoided;[49] and (ii) endogenous epitope presentation avoids potential low affinity, poor specificity, exhaustion, and/or anergy/tolerance problems associated with the excessively high density of epitopes presented following addition of exogenous peptide epitopes.[45,50,51] The additional advantage of using a vectored vaccine is the ability to easily co-deliver immunomodulatory cytokines.

Polytope vaccines against Epstein-Barr virus

There is now considerable indirect[52] and some direct evidence[53] that CTL provide immune control of EBV-infected B cells. A vaccine capable of inducing EBV-specific CTL is therefore likely to protect against both infectious mononucleosis (IM) and post-transplant lymphoproliferative disease (PTLD): diseases which are characterized by uncontrolled expansion of EBV-infected B cells.[52-54] The potential oncogenic capability of EBV[52,55] is likely to preclude a whole virus vaccine-based approach for EBV vaccination. Vaccines based on recombinant EBV nuclear antigens (EBNA), the targets of protective CTL immunity, carry a similar risk as individual EBNA proteins also have oncogenic potential.[52,56,57] Furthermore, any single EBNA protein does not appear to provide sufficient CTL epitopes to cover the HLA diversity of a target population.[58] These considerations make a compelling case for the epitope-based approach for an EBV CTL vaccine. A large number of epitopes have now been identified and simple calculations show that a polytope vaccine containing only seven epitopes restricted by HLA A2, A3, A11, B7, B8, B35 and B44 would protect >90% of a Caucasian population. One might postulate that a vaccine against IM or glandular fever only needs to stimulate the production of a small memory pool of EBV-specific CTL. Seronegative adolescents who are infected by EBV only have an approximately 50% chance of developing IM, suggesting that a few EBV-specific memory CTL might tip the balance towards asymptomatic seroconversion. Only a small number of adoptively transferred EBV-specific CTL were sufficient to clear an X-ray-visible PTLD.[53]

A polytope vaccine against melanoma

Cytotoxic T lymphocyte epitopes delivered by DNA vaccination have given therapeutic and prophylactic protection against tumours in animal models,[39,59] as have CTL epitopes

delivered by vaccinia.[12,37] Furthermore, human trials using peptide epitope-based therapeutic vaccines have shown tumour regression in melanoma patients.[37,60] These trials, therapy involving adoptive transfer of tumour infiltrating lymphocytes (TIL),[61] and the extensive characterization of melanoma-specific CTL, all point to the potential for CTL-based therapeutic melanoma vaccines.[62-64]

A multi-antigen CTL vaccine for melanoma would have several theoretical advantages. First, by simultaneously attacking multiple antigen targets, such a vaccine might limit immune evasion by tumours and/or metastases which have reduced expression of individual target antigens. Melanomas and their metastases vary considerably in target antigen expression: for example, 36% of primary melanoma and 76% of metastases express MAGE 3[65] and tyrosinase is expressed by 50% of melanomas. Checking of tumours in individual patients for expression of individual target antigens might also be avoided with a vaccine that contained multiple antigens.[60] Second, such a vaccine might prime for protective CTL responses directed towards antigens not previously recognized by a particular patient. This would diversify the patient's repertoire of CTL specificities capable of destroying melanoma cells. Third, as more melanoma antigens are identified, a cocktail of antigens containing epitopes restricted by multiple HLA alleles might be used to limit immune evasion by melanoma cells, which mutate individual epitopes and/or down-regulate individual HLA alleles.[66] Complete down-regulation of all HLA alleles should render the melanoma cell susceptible to NK/LAK lysis. The melanoma antigens recognized by CTL might be divided into melanoma differentiation antigens (tyrosinase, MART-1, gp100), self antigens aberrantly expressed (MAGE family) and altered self antigens (N-acetyl-glucosaminyltransferase V).[67] The relative therapeutic benefit of generating responses directed at each of these groups of epitopes remains to be established. The former two represent normal, non-mutated self sequences, and a vaccine increasing responses against these antigens has the potential to induce autoimmunity. The development of vitiligo (killing of normal melanocytes resulting in white areas of the skin) correlates with good prognosis in both melanoma and with adoptive transfer of TIL. Such autoimmunity might be considered acceptable, even favourable; however, potentially harmful effects on retinal melanocytes, although not yet observed in humans, needs to be carefully monitored.[63,64] MAGE (which is expressed on several tumour types) is also expressed in testis and placenta; whether autoimmune responses against these tissues occur, or will be considered serious, needs to be established.

The polytope strategy may simply offer a considerable reduction in the size and complexity of the vaccine product compared with a melanoma vaccine, which was designed to co-deliver multiple melanoma antigens using multivalent-single[64] or multiple-single antigen constructs. The potential additional requirement to co-deliver cytokines would further increase the size or number of constructs in vaccines based on the latter strategies. The polytope approach would also have the potential advantage of avoiding immunization with self sequences which might induce autoimmune CD4 T cell or antibody responses.

Simple priming of new CTL responses is unlikely to be the only activity a therapeutic melanoma vaccine will need to

induce in order to successfully regress established tumours. Many melanoma patients already have CTL responses, unlike mice bearing the model B16 melanoma.[69] Unfortunately, melanoma-infiltrating CTL in humans appear to be inactivated, anergized or may even be killed by the melanoma and/or its cytokine micro-environment.[70-72] Cytokine co-delivery may ameliorate some of these problems and a case has been made for co-delivering granulocyte/macrophage colony-stimulating factor (GM-CSF),[48,73] IL-2[74] and/or IL-12.[75] Conceivably a melanoma CTL vaccine needs to offer melanoma-specific CTL an opportunity to see peptide outside an immunosuppressive environment resulting in CTL proliferation and/or the CTL becoming de-anergized and again capable of killing melanoma cells.

Polytope vaccines against HIV/AIDS

There is now a considerable body of compelling indirect evidence that CTL have a role in preventing or limiting (or even clearing): (i) initial HIV infection; and (ii) progression to AIDS.[76,77] Interpretation of some of this evidence has been clouded by the discovery of the chemokine coreceptors, CXCR4/fusin and CCR3/5, for T cell and macrophage tropic HIV-1 viruses, respectively,[78] and a mutation of CCR5, which provides homozygotes with protection against infection.[79] Despite these developments, many observations still provide convincing evidence for the protective role of CTL in HIV/AIDS.[76,77,80-83]

Whole HIV vaccines, although being considered[84] are likely to face considerable safety and ethical hurdles.[85] HIV DNA vaccines based on single and multiple recombinant antigens are already being considered,[86] however, each antigen usually has several sequence variants with distinct CTL epitopes.[87] A recombinant protein-based vaccine covering most of the common epitope variants within one geographical region and containing enough epitopes to cover the HLA diversity of the population, would need to contain a considerable number of recombinant antigens. The number of epitope variants do not, however, appear to be limitless and are often restricted to conservative substitutions in 1 to 3 positions, suggesting that constraints are imposed on variation. Whether the polytope approach can be used to generate specific CTL responses to closely related epitopes in the face of potential competition and antagonism[88] remains to be tested.

The phenotype of vaccine-induced CTL responses is likely to be critical for an HIV vaccine. A drift from Th1 to Th2 (or Tc1 to Tc2) is associated with progression of HIV to AIDS[89] and recent studies have shown that CTL from long-term non-progressors (LTNP) maintain their capacity to produce IL-2, IFN-γ and IL-10 whereas CTL from progressors are unable to secrete IL-2 and IL-10 and synthesize IL-4.[90,91] The desired phenotype of vaccine-induced HIV CTL, should thus be largely Tc1, which might be achieved by co-administration of IL-12 or IFN-γ with the vaccine.[75,92] The chemokines RANTES, MIP1α and MIP1β, and SDF-1 are able *in vitro* to block the CCR3/5 and CXCR4 coreceptors, respectively, and thereby inhibit HIV infection. Induction of CTL secreting these chemokines may thus be an important goal for prophylactic and therapeutic HIV vaccines.[93] The activities of these chemokines *in vivo*, however, have yet to be fully resolved. They may have beneficial roles unrelated to physical blocking of HIV binding, associated with their ability to recruit and enhance CTL activity.[94] Another important factor secreted by CD8 CTL may be the so-called CD8 T cell antiviral factor (CAF), an elusive factor which inhibits HIV replication.[95] The identity of this factor has yet to be established but it appears not to be a standard chemokine.

A prophylactic HIV CTL-based vaccine would clearly need to generate CTL responses to all the epitope variants found within a particular target population and cover a substantial percentage of the HLA alleles within that population. The oral vaccination route would also be preferable as the virus enters via the mucosa[96] and long-term mucosal CTL memory can be maintained following oral vaccination.[97] Progress in oral DNA vaccine delivery might soon be expected. The enthusiasm for a therapeutic HIV vaccine may have diminished following the recent successes in drug treatment, however, such drugs might eliminate HIV only if administered for a lifetime. This would be expensive and is likely to select for resistance. A vaccine which restored, broadened and strengthened cellular immunity, perhaps associated with drug therapy, may have the best chance of providing long-term benefit.[98]

The recent demonstration of CTL escape mutants[81,83] also strongly suggests that inclusion of multiple variants in a therapeutic vaccine might pre-empt potential escape mutants, and may thereby convert individuals who progress to AIDS into LTNP.

Vaccination modalities capable of inducing CTL in humans

A large number of vaccination modalities have been developed which are capable of delivering recombinant proteins for the induction of CTL; these include viral and bacterial vectors, liposomes, microspheres, virus-like particles, ISCOM, synthetic peptides and DNA. At present, principally because of safety and quality control issues, only a limited number of modalities have entered phase I human clinical trials with the aim of inducing protective CTL specific for vaccine antigens or epitopes. These modalities, which include poxvirus vectors,[99] synthetic peptide formulations,[47,48,100] Ty particles[101] and DNA vaccination,[102] will be competing to be the modality of choice for CTL induction in humans.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Suhrbier, et al.	Group Art Unit: 1644
Serial No.: 09/576,101	Examiner: Huynh, P. N.
Filed: May, 22 2000	Atty. Dkt. No.: FBRC:004USC1/TMB
For: POLYEPITOPE VACCINES	

Appendix F

DECLARATION OF JOHN COOPER COX AND EXHIBITS JCC1-JCC17

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Suhrbier, et al.	Group Art Unit: 1644
Serial No.: 09/576,101	Examiner: Huynh, P. N.
Filed: May, 22 2000	Atty. Dkt. No.: FBRC:004/TMB
For: POLYEPITOPE VACCINES	

DECLARATION OF JOHN COOPER COX

I, John Cooper Cox, hereby declare as follows:

1.   I am an Australian citizen residing at 140 Dunns Road, Bullengarook, Victoria 3437 Australia. I am a Consultant Scientist of CSL Limited, an assignee of the Applicants' invention.

2.   I have extensive experience in the field of chemistry, biochemistry, immunology, virology, vaccine delivery, AIDS research, and bacteriology. References containing

examples of my work are included in my *Curriculum Vitae*, a copy of which is affixed hereto as Exhibit JCC1.

3.   I understand that The Present Application was filed on May 22, 2000 as a continuation application of USSN 08/776,337 filed April 21, 1997 which is a US national phase application of International application No. PCT/AU95/00461 filed on July 27, 1995. The International application claims priority from Australian Patent Application No. PN1009 filed February 8, 1995 and from Australian Patent Application No. PM7079 filed July 27, 1994.

4.   I consider myself to have been in possession of at least ordinary skill and knowledge in the arts relevant to this invention when the invention was made (i.e. before July 27, 1994) and at all times until The Present Application was filed on May 22, 2000.

5.   I have read and understood the specification and pending claims of the above-captioned application ("The Present Application") and an Office Action by the United States Patent and Trademark Office mailed on May 21, 2002 in connection with The Present Application. I have also read the revised claims of The Present Application which I understand the applicants have submitted in response to the May 21, 2002 Office Action. A copy of the revised claims is affixed hereto as Exhibit JCC2.

6.   I make this declaration in response to the Examiner's rejection of pending Claims 14-34 of The Present Application at paragraphs 8 and 9 of the Office action mailed on May 21, 2002. I understand that the Examiner has rejected The Present Application on the ground that the specification as originally filed failed to provide a sufficient written description of the invention as claimed to reasonably convey to one skilled in the art that the inventors were in possession of the claimed invention when the application was filed. I understand from the Examiner's comments at paragraph 8 of the Office Action that the specification must "clearly allow persons of ordinary skill in the art to recognize that [the inventors] invented what is claimed" [emphasis added].

7.   I will begin by first summarizing my understanding of the invention in the context of what was already known before the first US application by the inventors was filed, then

analyzing the allegations made by the Examiner in the May 21, 2002 Office Action in light of what the inventors claim as their invention in The Present Application. In this respect, it is my understanding that if there are any omissions from the specification that would be required to convince a skilled person that the inventors had actually invented what is claimed, those omissions may be provided by the prior art as it stood before the filing date of The Present Application. I also understand that The Present Application must provide sufficient representative examples for a skilled person to conclude that the inventors invented the full scope of subject matter claimed in The Present Application.

The Invention in Context of The Prior Art

8. For more than 10 years prior to the filing date of The Present Application, there had been a growing understanding of the importance of cytotoxic T-cell lymphocyte (CTL) responses in preventing infection, enhancing resistance to clinical disease, and assisting recovery following infection or reinfection. As a result of the significance of CTL responses in mediating immunity, considerable effort was made to identify the peptide epitopes involved in mediating CTL responses to a range of different microbial pathogens and cancers. As stated at paragraph 3 of The Present Application, these peptide epitopes ("CTL epitopes") are mainly generated by proteolysis in the cytosol, and transported to the endoplasmic reticulum for association with the major histocompatibility complex (MHC).

9. Before the filing date of The Present Application, and even before 1994, it was known that the structure of a CTL epitope is a peptide of about 9 amino acids in length, however a CTL epitope may occasionally consist of 7-10 amino acids in length. It was also known at that time that the CTL epitope peptide is incorporated within the groove in the major histocompatability complex type 1 (MHC-1) surface antigen on antigen presenting cells (APC) and then "presented" to $CD8^+$ T cells, otherwise known as cytotoxic T lymphocytes (CTL). CTLs were also known then to be distinct from $CD4^+$ helper T-cells. The groove in the MHC-1 surface antigen is closed at both ends, thereby restricting the upper limit on the length of a CTL epitope that can interact with this groove. A CTL epitope which will fit within this groove without further modification is called a

"minimal CTL epitope". The size of a CTL epitope was established by two different methodologies:

(i)   elution of peptides from the class I MHC of APCs; and

(ii)   X-ray crystallography which demonstrated that 7-10 amino acids can fit into the MHC-1 groove.

These concepts are described in detail by Rammensee *et al.*, *Ann. Rev Immunol. 11*, 213-244, 1993 (Exhibit JCC3).

10.   The principles of CTL recognition were initially established in murine systems. Subsequent experience demonstrated that the principles established in murine systems also applied to human cells (e.g., Townsend *et al.*, *Cell 44*, 959-968, 1986; Exhibit JCC4). Townsend *et al.* provided the first definition of the concept that CTL recognised short peptide fragments and that both murine and human CTLs recognize degraded peptide fragments of proteins in association with MHC Class I molecules. In my opinion, the demonstration by Townsend *et al.* that CTL epitopes of Influenza Virus nucleoprotein in association with MHC Class I molecules were recognized by similar mechanisms in mouse and man clearly establishes the relevance of murine models in assessing immunological responses. The difference lies only in the detailed selection of the CTL epitopes and not in the mechanism by which the immune response recognizes a particular epitope. This is because few CTL epitopes will be recognized by both murine and human CTLs. However, as is apparent from the following paragraphs, I do not consider that the selection of specific CTL epitopes is a major obstacle, even in 1994. The concept of the general mechanistic similarity between murine and human CTL presentation is also referred to by Rammensee *et al* 1993 (Exhibit JCC3).

11.   Before the filing date of The Present Application, various groups of workers had identified CTL epitopes in a number of different disease conditions including hepatitis B, human papilloma virus, human immunodeficiency virus, Epstein-Barr virus and human melanoma. As a person skilled in the art of immunology and vaccine technology, I am aware of several examples of such CTL epitopes (*e.g.*, Khanna *et al.*, *J. Exp. Med 176*, 169-176, 1992 (Exhibit JCC5) and Moss *et al.*, *Adv. Cancer Res. 69*, 213-245, 1996

(Exhibit JCC6) described CTL epitopes from EBV; and Toes *et al., Proc. Natl Acad. Sci (USA) 94,* 14660-14665, 1997 (Exhibit JCC7) describe CTL epitopes against tumor antigens). Rammensee *et al., Ann. Rev Immunol. 11,* 213-244, 1993 (Exhibit JCC3) also describe a total of 58 CTL epitopes, representing only a fraction of CTL epitopes known at that time. I also note that in the Office Action mailed on May 21, 2002, the Examiner also acknowledges several prior art CTL epitopes from a wide range of pathogens and tumor antigens, such as, for example, the following CTL epitopes:

(i)   a CTL epitope from CMV and a recombinant vaccine comprising same as described by Del Val *et al., J. Virol. 65,* 3641-3646, 1991;

(ii)   a CTL epitope from Influenza Virus as described by Latron *et al., Proc. Natl Acad. Sci (USA) 88,* 11325-11329, 1991;

(iii)   CTL epitopes from EBV as described by Burrows *et al., J. Gen. Virol. 75,* 2489-2493, 1994; and

(iv)   CTL epitopes from the melanoma tumor antigens MAGE-1 and MAGE-2 and its potential use in vaccines, as described by Celis *et al., Proc. Natl Acad. Sci (USA) 91,* 2105-2109, 1994 and references cited therein at paragraph 1 of Celis *et al.*

12.   The specification of The Present Application also discusses in a general sense CTL epitopes from one or more pathogens or tumor antigens, and exemplifies CTL epitopes from human EBV (Table 1) and from a range of murine pathogens as listed in Table 2, specifically Influenza Virus, Adenovirus, Sendai Virus, *Plasmodium berghei*, CMV and LCMV.

13.   Before the filing date of The Present Application, I was also aware of several well-established methods for determining CTL epitopes from any source. For example, predictive algorithms may provide some indication of the presence of a CTL epitope in an antigen, however such methods are in no way definitive. CTL epitopes are also known to be defined in a chromium release assay wherein the minimal amino acid sequence capable of making a target cell susceptible to CTL (usually CD8+) lysis is determined. Target cells can be transfected with the gene coding for the protein which comprises the

epitope of interest or alternatively the target calls can be exposed to the high concentrations of the minimal epitope. Generally, target cells are labelled with radioactive chromium and mixed with activated T cells, and the extent of lysis is measured by the release of soluble chromium. This methodology precedes 1994 and is accepted by all immunologists. By 1997, numerous minimal CTL epitopes had been defined by chromium release assay (e.g., Rickinson and Moss, *Ann. Rev. Immunol. 15*, 405-431, 1997; Exhibit JCC8). Additionally, the ELISPOT assay enumerates the strength of a memory T cell response to a given CTL epitope, via a measurement of interferon (IFN)-gamma. As well as measuring the precursor frequency to defined CTL epitopes, the ELISPOT assay is used to scan peptide sequences to define new CTL epitopes. As far as I am aware, Miyahira *et al., J Immunol Methods 181,* 45-54, 1995 (Exhibit JCC9) provide the first disclosure of the use of ELISPOT in elucidating a minimal CTL epitope. Additionally, Class I Tetramer assays elucidate CTL structure by visualizing CTL epitope binding (e.g., by FACS) to a T-cell receptor on CTLs (Altman *et al Science 274,* 94-96, 1996; Exhibit JCC10). The advantage of Tetramer assays is that they allow both the enumeration, characterisation and isolation of CTLs that are reactive with a specific CTL epitope peptide.

14. CTL epitopes were also known before the present invention and filing date of The Present Application to be MHC-restricted. What this means is that a given CTL epitope will only be recognized by individuals with the same HLA allele in their genetic background. By 1995, various groups had identified extensive lists of CTL epitopes of known HLA restriction.

15. In view of the known MHC restriction of CTL epitopes, any single formulation capable of inducing a CTL response in a genetically diverse group of individuals will need to contain one or preferably several epitopes for each of the normally encountered HLA groups within the population. Typically around 10 to 20 or more individual CTL epitope peptides may be required within such a formulation. In my considered opinion, it was a reasonably straight forward exercise before the present invention and well before the filing date of The Present Application to select a subset of known CTL epitopes having

known MHC restriction which would be recognized collectively, by a broad cross-section of a population.

16. Notwithstanding that the selection of suitable CTL epitopes was routine, a problem facing workers even in 1994 was how to incorporate a large number of CTL epitopes into a single formulation that would achieve a significant population coverage. This is because CTL epitopes vary considerably in their degree of hydrophobicity and in turn their aqueous solubility. Most workers attempted to present single epitopes within a water-in-oil emulsion, typified by Freunds incomplete adjuvant (IFA). Around this time, I was involved in such studies and concluded that these emulsions were the preferred formulation (see Scalzo *et al*, *J. Virol.* *69*, 1306-1309, 1995). However, later studies showed how difficult it was to extend these findings to formulations comprising a large number of CTL epitope peptides.

17. From my reading of the specification as originally filed, The Present Application addresses the problem of providing a single formulation comprising a large number of CTL epitopes, by providing a plurality of minimal CTL epitopes in the form of a single fusion protein (i.e a "polyepitope" polypeptide). This approach was new because, as stated at paragraph 3, lines 7-8, of The Present Application, the "influence of sequences flanking CTL epitopes on the proteolytic processing of these epitopes remains controversial". I agree with this conclusion at paragraph 3 of The Present Application because it clearly was not known before the invention how to produce a single polypeptide comprising multiple minimal CTL epitopes. I understand that polyepitope polypeptides described in The Present Application comprise a plurality of CTL epitopes wherein each CTL epitope is correctly processed, and presented to APCs such that each minimal CTL epitope induces a CTL response, even though the CTL epitopes are not flanked by naturally occurring flanking sequences or an initiation codon (i.e. methionine) in polypeptide formulations.

The Invention Claimed in The Present Application

18. I have confined my remarks herein to the amended claims of The Present Application (Exhibit JCC2).

<div align="center">The Polynucleotide Claims</div>

19. The Examiner states at paragraph 8 of the May 21, 2002 Office Action that the specification fails to provide an adequate written description of polynucleotides encoding a plurality of CTL epitopes, specifically:

    (a)   the structure associated with the function of any polynucleotide encoding a plurality of CTL epitopes; and

    (b)   a representative number of polynucleotides encoding a plurality of CTL epitopes other than those murine CTL epitopes and B cell epitopes listed in Table 2, as expressed in the vaccinia viral vector depicted in Figure 5.

20. At paragraph 9 of the Office Action, the Examiner also rejects Claims 14-34 on the basis that the specification as originally filed requires the CTL epitopes of the polyepitope polypeptide to be "substantially free of sequences encoding peptide sequence naturally found to flank the CTL epitopes". I understand from the amended claims (Exhibit JCC2) that this feature has now been introduced to the claims.

21. Claim 14 is directed to a polynucleotide that encodes a single fusion polypeptide comprising a plurality of CTL epitopes. In my experience, the normal and customary meaning of the word "plurality" in the context of the present invention would be that more than one epitope is present, and does not act to exclude any particular number of epitopes.

22. Methods described in the specification for producing such a polynucleotide include splicing together of synthetic oligonucleotides by overlap extension (SOEing) and PCR (paragraphs 36 and 45). In my opinion, it was a simple matter for any skilled molecular

biologist even in 1994 to produce a nucleic acid sequence encoding a specific amino acid sequence when provided with the amino acid sequence and the genetic code, and perhaps codon preference data. The inventors were clearly aware of such technology, as they state at paragraph 45, lines 6-7, of the subject specification "two groups of epitopes were converted to a DNA sequence using the universal codon usage data".

23. The requirement in amended Claim 14 for each CTL epitope to be "substantially free of sequences encoding peptide sequences naturally found to flank that CTL epitope" is explained at paragraph 5 of the subject specification. I understand that each CTL epitope of the polyepitope polypeptide comprises a minimal CTL epitope and possibly, in addition, a small length (i.e. 1-5 amino acids) found naturally to flank the minimal CTL epitopes. This means that each CTL epitope will have a length not exceeding 15 contiguous amino acids of the antigen from which it is derived. Clearly, The Present Application exemplifies such epitopes in Tables 1 and 2. As a skilled immunologist, the lack of naturally occurring flanking sequences or start codons/methionine in the sequences flanking the CTL epitopes in the polyepitope constructs of the invention does not depend upon the particular CTL epitopes employed.

24. Claim 14 also requires the absence of any intervening methionine in the encoded polyepitope polypeptide. As would be known to a skilled molecular biologist, a methionine in a polypeptide is encoded by a "translation initiation codon" (i.e. the codon ATG) in the corresponding nucleic acid. For expressing a fusion polypeptide, it is well known that there should not be an intervening translation initiation codon in the corresponding nucleic acid sequence, otherwise that codon might be used to initiate translation at an inappropriate internal site. Conversely, by omitting the internal translation initiation codon from the polyepitope polypeptide of The Present Application, the inventors have ensured that the plurality of CTL epitopes are expressed as a single polypeptide. In the context of the polynucleotide structure, this means that nucleic acid sequences encoding the minimal CTL epitopes, and any intervening sequences if present, would need to be presented as a single open reading frame, positioned between a single upstream promoter sequence and a single downstream transcription terminator sequence. Additionally, a single translation initiation codon would be necessary at the

commencement of the open reading frame for the CTL epitopes. These features are described, for example, at paragraph 45 of The Present Application. This "polyepitope" arrangement is distinct from the "minigene" arrangement as described by Whitton *et al.*, *J. Virol. 67*, 348-352, 1993 wherein individual CTL epitopes are each preceded by their own translation initiation codon.

25. Based upon the disclosure in the specification and what was known before the filing date of The Present Application, I disagree with the Examiner's conclusion that there are insufficient examples of the claimed polynucleotides to demonstrate that the inventors were in possession of their invention at that time. The features recited in newly amended Claim 14 are generally supported by the description in The Present Application, at paragraphs 3 to 6. As the prior art disclosed a wealth of CTL epitopes as acknowledged by the Examiner in the May 21, 2002 Office Action, and the specification of The Present Application also disclosed CTL epitopes from the antigens of human and murine pathogens, the specification contains an adequate written description of CTL epitopes. A person skilled in the art would certainly have no difficulty in performing the invention claimed for want of CTL epitopes.

26. As one skilled in the art, I can also state that the artisan would find the matter of making the constructs of the present invention, armed with the guidance provided in the specification, a routine matter. The materials and general molecular procedures for constructing the polyepitope constructs were readily available to the artisan at the time the application was filed. Moreover, given the plethora of CTL epitope sequences known and available at that time, the general knowledge of a skilled molecular biologist, and the fact that The Present Application exemplifies two divergent polynucleotides encoding such fusion polypeptides (Examples 1 and 2), I have no difficulty in accepting that the inventors had actually reduced to practice a general method as claimed for producing a polynucleotide encoding a single fusion polypeptide comprising multiple CTL epitopes substantially free of sequences encoding peptide sequences naturally found to flank the CTL epitopes.

27. Claims 14 and 15 further define a situation wherein two or more of the multiple CTL epitopes are joined end-on-end without intervening sequences there between (i.e. they may be contiguous). In my opinion, there is clear support for the feature of contiguous epitopes *inter alia* in Examples 1 and 2 of The Present Application (*see* paragraphs 36 through 44).

28. Example 1 of the specification as originally filed taught a construct encoding a polyepitope consisting of nine HLA-restricted (i.e. human) EBV CTL epitopes as listed in Table 1 of the specification. The nine CTL epitopes were flanked by two B cell epitopes. The polynucleotide encoding a single polypeptide consisting of the nine CTL epitopes and two B cell epitopes incorporated a single Kozac consensus sequence and a single translation start site upstream of the open reading frame encoding the epitopes (i.e. there was no internal translation initiation codon between the sequences encoding the CTL epitopes). The CTL epitopes were each only 9-10 amino acids in length and, as a consequence, the polynucleotide encoding the CTL epitopes meets the requirement of being substantially free of nucleic acid sequences encoding naturally occurring flanking sequences (*see* final two lines of paragraph 36 and the description of Table 1). The construct was cloned into vaccinia virus and used to infect target cells, which expressed the HLA alleles restricting each epitope. Autologous CTL clones specific for each epitope were then used as effector cells. The positive results presented in Figures 2 and 3 of the specification indicated that each epitope in the expressed human polyepitope was processed within the target cells and recognized specifically by human CTL clones. The construct was also cloned into pGEX-3X and used to transfect *E. coli* cells. A fusion protein of the expected molecular weight was identified in a Western blot using antibodies against the B cell epitopes, further supporting correct expression of the complete fusion protein (i.e. polyepitope).

29. Example 2 of the specification as originally filed taught a construct encoding polyepitopes consisting of 10H-2 restricted (i.e. mouse) CTL epitopes, including epitopes derived from Influenza Virus, Adenovirus, Sendai Virus, Ovalbumin, *Plasmodium berghei*, murine CMV, and LCMV. As shown in Figure 5, the exemplified fusion polypeptide consisted of ten CTL epitopes, arranged in two groups of five contiguous

epitopes separated by intervening non-epitope spacer sequence TS. The two groups of five contiguous CTL epitopes had the following amino acid sequences:

(i)   ASNENMDAM-SIINFEKL-TYQRTRALV-SDYEGRLI-YPHFMPTNL; and

(ii)   SGPSNTPPEI-FAPGNYPAL-SYIPSAEKI-EEGAIVGEI-RPQASGVYM.

At the C-terminal end of the CTL epitopes, a second spacer having the sequence PR was positioned to separate the CTL epitopes from a B cell epitope (NNLVSGPEHL). The polynucleotide encoding a single polypeptide consisting of the ten CTL epitopes and one B cell epitope incorporated a single Kozac consensus sequence and a single translation start site upstream of the open reading frame encoding the epitopes (i.e. there was no internal translation initiation codon between the sequences encoding the CTL epitopes). The CTL epitopes were each only 8-10 amino acids in length and, as a consequence, the polynucleotide encoding the CTL epitopes meets the requirement of being substantially free of nucleic acid sequences encoding naturally occurring flanking sequences. The constructs were cloned into vaccinia virus and used to infect mice. Three different strains of mice were infected with constructs, and these strains mounted CTL responses to each of the appropriately-restricted epitopes in the expressed polyepitopes. From the information provided in The Present Application when it was originally filed, specifically the different combinations of CTL epitopes from murine and human antigens exemplified in the application, I conclude that the inventors were in possession of this feature as claimed generically in Claim 14.

30.   Example 2 also taught that the CTL epitopes from various pathogens in the polyepitope generated primary CTL responses to each epitope. Example 2 of the specification as originally filed taught that the CTL responses induced by the polyepitope was, in fact, protective. This analysis was performed using a tumor model (i.e. ovalbumin-transfected EL4 cells in C57BL/6 mice) and in Balb/c mice infected with MCMV (paragraph 56).

31. The specification also teaches that multiple epitopes can be effectively processed and presented within a single host even where the epitopes are restricted by a single HLA allele. Such processing has not been shown previously and is a highly desirable feature for both prophylactic vaccines, and is probably essential for therapeutic vaccines.

32. In my considered opinion any skilled immunologist would readily appreciate from the teachings of the specification that the claimed polyepitope constructs could comprise two or more epitopes so conjoined as to exclude naturally occurring flanking sequences or start codons/methionine. Therefore, constructs comprising three, four, five, six, seven, eight, nine, and at least ten CTL epitopes are indicated by the specification. Therefore, the demonstration that at least ten such epitopes are successfully processed, *etc.*, in light of the Inventors' statement that a plurality of epitopes may be so conjoined, would reasonably lead one of skill in the art to conclude that the Inventors contemplated at least three, and potentially any number of epitopes be conjoined according to the invention.

33. Claim 14 also defines a situation wherein at least two CTL epitopes in the polyepitope are separated by a non-natural spacer (i.e. an "inter-epitope spacer"), provided that the spacer does not include a translation start methionine. In my opinion, this embodiment is clearly supported by the exemplification provided in Figure 5, which shows a spacer sequence TS between the epitopes YPHFMPTNL and SGPSNTPPEI. By definition, at least a fragment of each internal CTL epitope in the polyepitope acts a spacer between flanking CTL epitopes. For example and with reference to Figure 5, at least a part of the epitope SIINFEKL can be considered to act as an inter-epitope spacer between the epitopes ASNENMDAM and TYQRRTRALV. Proceeding on this basis, it is apparent that the inventors have, in fact, exemplified a range of different inter-epitope spacers. From the information provided in The Present Application when it was originally filed that there is sufficient exemplification of this feature to conclude that the inventors were in possession of this feature as claimed generically in Claim 14.

34. Thus, the inventors have demonstrated that a polynucleotide construct can be used to express a polyepitope protein, that is processed to the individual component epitopes which are then each expressed on the cell surface and induce CTL-responses thereby killing infected cells. Accordingly, The Present Application indicates to me that the inventors arrived at a general method whereby CTL epitopes (i.e. peptides of 9 ± 1 amino acids) are joined end to end contiguously, or alternatively, with spacing amino acids not normally associated with the epitopes in their native context. The resulting polyepitope polypeptide, when formulated appropriately, is processed within the cytoplasm of an antigen presenting cell (APC) so that the identity of the individual CTL epitopes is preserved. The induction of an *in vivo* CTL response to these epitopes is proof of the preservation of their identity.

35. The Present application further indicates to me that multiple CTL epitopes of the polyepitope polypeptides that are restricted by the same HLA type (or in the case of the mouse, the H2 type) can be processed and presented effectively at the same time.

36. As a person having considerable skill in the immunological arts, The Present Application makes it possible to formulate a vaccine (either polynucleotide or polypeptide) which will contain sufficient antigenic content to induce CTL responses to one or more proteins within a pathogen or a tumour over a broad cross-section of the human population. Prior to The Present Application, even though the potential importance of CTL responses had been recognized and many CTL epitopes had been identified, the only proposed way to formulate these epitopes was as individual epitopes in a water-in-oil (eg Freunds) emulsion. Such a formulation did not require intracellular processing. The Present Application has shown for the first time that a single strand of DNA or a single polypeptide can contain all the information required to induce a broad anti-tumour or anti-pathogen CTL response in essentially all members of a human population.

37. At paragraph 9 of the May 21, 2002 Office Action, the Examiner also rejects Claims 16, 18 and 19 on the basis that the specification as originally filed fails to provide a clear support for "at least two", "nine" or "ten" CTL epitopes.

38. Claims 16 through 19 as amended specify the number of individual CTL epitopes encoded by the polynucleotide. Specifically, the claims define "at least three", "four", "nine" and "ten" CTL epitopes. As will be apparent from the preceding discussion, Examples 1 and 2 of The Present Application as originally filed teach polynucleotides encoding a polyepitope polypeptide consisting of nine and ten CTL epitopes, respectively. Support for "at least three CTL epitopes" is to be found *inter alia* at paragraph 10 of the specification. Support for "four CTL epitopes" is to be found *inter alia* at paragraph 74. The established success of single CTL epitopes in generating CTL responses, and the success of polyepitopes comprising at least three, four, nine or ten CTL epitopes as evidenced in The Present Application clearly indicates to me that any number of CTL epitopes could reasonably be provided in a single polyepitope polypeptide.

39. In my opinion, there is sufficient teaching provided in The Present Specification to indicate that the inventors were aware when the application was filed of how to conjoin any number of CTL epitopes substantially free of natural flanking sequences, from any number of different antigens from a number of different pathogens, particularly given the plethora of known CTL epitopes even as early as 1994-1995. The described constructs are generic rather than being limited to the exemplified polyepitope polypeptides because, if a set of minimal CTL epitopes are known, then the process for selecting a subset of epitopes is mechanical based upon their HLA restriction, and their formulation into a single polyepitope can be achieved following the teaching in The Present Application. For example, following the teaching provided in The Present Specification, a skilled person seeking to produce an HIV vaccine would select from the literature a range of minimal CTL epitopes from appropriate HIV proteins (e.g. *gag*, *env*, *nef*, etc) where multiple epitopes from each protein may be chosen to cover a range of HLA restrictions (e.g. A2, B8, A24, B35, etc.). The total number of CTL epitopes required to formulate an effective vaccine with a wide population coverage may easily exceed 30 or

even 40 epitopes. Accordingly, I am satisfied that the inventors have provided a sufficient exemplification of the effect of varying CTL epitope number in the claimed polyepitope to reasonably convey that they were in possession of the full scope of polyepitopes claimed.

40. The Examiner also alleges that the specification fails to provide an adequate written description of polynucleotides encoding a plurality of CTL epitopes derived from a plurality of pathogens.

41. Claims 25 through 28 specify the source of the CTL epitopes as being from a single pathogen (claim 25), multiple pathogens (claim 26), the specific pathogens Epstein Barr Virus (EBV), Influenza Virus (IV), Cytomegalovirus (CMV), Adenovirus (Ad), and human Immunodeficiency Virus (HIV) (Claim 27), or a tumor protein (Claim 28). In this respect, I note that Example 1 teaches constructs encoding polyepitopes comprising epitopes of EBV, and that Example 2 teaches constructs encoding polyepitopes comprising epitopes of IV, Ad, Sendai Virus, LCMV, *Plasmodium berghei,* and CMV and a surrogate tumor epitope (i.e. the SIINFEKL epitope in the ovalbumin protein). Although no specific HIV CTL epitopes are described, reference is made to HIV epitopes at paragraph 51 of the specification. Given the plethora of known CTL epitopes from HIV at the filing date of The Present Application, and from the teaching provided that individual CTL epitopes are correctly processed and presented from the claimed polyepitope formulation, I conclude that it would be a routine matter to substitute the exemplified CTL epitopes provided by the inventors with other CTL epitopes, specifically known HIV-1 CTL epitopes. Accordingly, the specification as originally filed reasonably conveys that the inventors were in possession of polynucleotides encoding polyepitope polypeptides comprising CTL epitopes from a wide range of pathogens.

42. Claims 29 through 32 are directed to the polynucleotide of claim 14 comprising as an additional feature a nucleic acid sequence encoding a $CD4^+$ T helper epitope, a B cell epitope, or a toxin. The description at paragraph 12 of The Present Application clearly recites the presence of such additional features. Examples 1 and 2 of the specification

clearly teach polyepitopes comprising B cell epitopes, as discussed *supra*. In view of the accepted use of such features in vaccine technology, and the exemplified use of CTL epitopes in conjunction with B cell epitopes, I do not have any doubt that the inventors were in possession of these features when the application was filed.

43.   The Examiner also asserts that the only use of the constructs of the present invention is as a vaccine in the prevention of particular diseases. Accordingly, the Examiner appears to be treating those claims directed to polynucleotides as somehow being "disguised" vaccine claims. I strongly disagree with this conclusion by the Examiner.

44.   In view of the various uses and methods provided in the specification, and the utility of the polyepitope-encoding constructs in the study of cellular and immune responses, I find this assertion by the Examiner to be incorrect. For example, the constructs can be used for the *ex vivo* stimulation of CTL clones (i.e. adoptive transfer), as described . As would be appreciated by those of skill in the art, the polyepitope constructs of the present invention have a broad utility as components of highly protective vaccines. However, such polyepitope-encoding constructs would also be known to have many uses beyond generating completely protective immune responses for particular diseases. Indeed, even as formulated as a vaccine, the constructs of the present invention would be useful in the study and modulation of immune responses of various degrees of protection. Moreover, as I discuss above, and as would be readily apparent to those of skill in the art, the constructs have additional uses in the study and manipulation of basic cellular and immunological phenomena.

The Vector Claims

45.   At paragraph 9 of the May 21, 2002 Office Action, the Examiner has rejected Claim 21 on the basis that there is no adequate written description in the specification of viral vectors other than vaccinia or avipox virus vectors.

46. Claims 20 through 24 define vectors comprising the polynucleotide of claim 14. Claim 20 is generic. Claims 21 and 24 define the species of a viral vector and a virus-like particle (VLP). Claims 22 and 23 define two species of viral vectors, namely vaccinia vectors and avipox vectors.

47. Paragraph 13 of the specification discusses the range of vectors contemplated, however only as *examples* of the full scope of vectors known at the filing date:

> "vaccinia vectors, avipox virus vectors, bacterial vectors, virus-like particles (VLPs) and rhabdovirus vectors, or by nucleic acid vaccination technology"

48. The specification further cites in the final lines of paragraph 13 the disclosures by Chatfield *et al, Vaccine 7,* 495-498, 1989 (Exhibit JCC11) and Taylor *et al., Vaccine 13,* 539-549, 1995 (Exhibit JCC12) and Hodgson "Bacterial Vaccine Vectors" in Vaccines in Agriculture (Exhibit JCC13). Clearly, a wide range of vectors were known well before the filing date of the Present Application.

49. Moreover, from my reading of the specification it is clear that the inventors were in possession of vaccinia vectors, bacterial expression vectors (e.g. a bacterial cell comprising the plasmid pGEX-3X), and the mammalian expression vectors described at paragraph 58 of the subject specification which utilize a CMV promoter or RSV promoter.

50. As a person skilled in the art of vaccine technology, I am also aware that well before the filing date of The Present Application the features of DNA vaccines most relevant for enhanced vaccination were well known. Numerous vaccine vectors were described which were capable of eliciting persistent humoral and cell-mediated immune responses to a wide range of viral, bacterial and parasite antigens. The vectors are generally non-replicating, non-infectious and non-integrating, and are stable and easy to prepare at low cost. Examples of such vectors are described for example, by Fynan *et al., Proc. Natl Acad. Sci (USA) 90,* 11478-11482, 1993 (Exhibit JCC14); Pardoll *et al., Immunity 3,* 1650169, 1995 (Exhibit JCC15); and Ramsay *et al., Immunol. Cell Biol. 75,* 382-388, 1997 (Exhibit JCC16). Fowlpox virus vectors were also well known before the filing

date of The Present Application, as described by Somogyi *et al.*, *Virol. 197*, 439-444, 1993 (Exhibit JCC17).

51. Having shown that each CTL epitope of a polyepitope is correctly processed and presented to APCs, and given the success of DNA vaccination with such a wide range of vectors before the filing date of The Present Application, the general teaching of vectors provided in the specification when it was originally filed is sufficient to adequately convey that the inventors were in possession of the claimed genus.

The Vaccine Claims

52. The Examiner also alleges that the specification fails to provide an adequate written description of polynucleotides encoding a plurality of CTL epitopes that are useful for a vaccine preventing disease. The Examiner also suggests that the specification only provides sufficient information and guidance to contemplate a use for the constructs to achieve completely protective vaccination

53. Claims 33 and 34 are directed to nucleic acid vaccines. The claimed vaccines comprise a polynucleotide having identical properties to the polynucleotides of claims 14 and 15, respectively.

54. I strongly disagree with the Examiner's conclusion in respect of Claims 33 and 34. As to the Examiner's suggestion that the specification only provides sufficient information and guidance to contemplate a use for the constructs to achieve completely protective vaccination, I disagree with the Examiner's assessment of both the scope of the teachings of the specification and the uses to which the polyepitope constructs may be put.

55. As I understand it, the relevant question with regard to such claims, as distinct from claims directed to mere polynucleotides, is their ability to elicit a protective immune response.

56. I note that at least one construct exemplified in The Present Application (i.e. Example 2) did confer a protective immune response. As noted *supra*, Example 2 of the specification

as originally filed taught that the CTL responses induced by the polyepitope was, in fact, protective. This analysis was performed using a tumor model (i.e. ovalbumin-transfected EL4 cells in C57BL/6 mice) and in Balb/c mice infected with MCMV (paragraph 56).

57. I have already stated herein above that I consider the specification to provide a sufficient disclosure of polyepitope-encoding constructs in view of the plethora of known CTL epitopes available at the filing date of The Present Application and the broad teaching in the specification for the first time as to how to formulate such CTL epitopes into a single polyepitope-encoding construct. Many of the known CTL epitopes at that time were potentially useful for vaccines to prevent disease. Furthermore, given the detailed description of the claimed invention in the specification and that the constructs provided by the specification are generic for constructs comprising two or more CTL epitopes, a skilled researcher in the field of vaccine delivery, bacteriology or virology, desirous of constructing a composition for use as a vaccine, would understand that the selection of appropriate epitopes for any particular vaccine application would be within the capabilities of the skilled researcher. The incorporation of such epitopes into a polyepitope construct as provided by the inventors would be routine in view of the skill in the arts and the guidance provided by the specification.

58. Given the similarity in the immune responses of mouse and man as discussed *supra*, and the fact that the inventors were actually able to demonstrate protective immune responses in mice, I conclude that the specification as filed did actually provide a sufficient representative number of examples of polyepitopes useful for a vaccine preventing disease. If the Examiner considers that the inventors should need to provide data on human vaccination trials, which would only be available in the form of phase I/II/III clinical trials, then in my opinion the Examiner has misconstrued the invention and not fully appreciated the general acceptance of mouse models in the immunological arts.

59. The Examiner may have failed to fully appreciate the benefits to be derived from the invention, which should not be underestimated. For example, the description at paragraph 75 clearly and unequivocally states that in the case of polyepitope-encoding

constructs designed to give protection against a variety of different diseases, "an individual receiving such a polyepitope vaccine, but who had already been exposed to one of the target diseases would still be immunized against the remaining CTL epitopes in the polyepitope". The impact of the finding by the inventors that each CTL epitope is correctly processed and presented therefore provides enormous benefits, including vaccinating human populations against a wide range of different pathogens using a single formulation, and significant cost benefits to the industry.

60. In summary, the specification as filed provided a sufficient teaching of the broad applicability of their invention in so far as it relates to a polynucleotide comprising a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) epitopes wherein each CTL epitope is substantially free of sequences encoding peptide sequences naturally found to flank that CTL epitope and wherein at least two of the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence that does not comprise a translation start methionine, to demonstrate that they were in possession of the invention. In my opinion, the invention has much broader applicability than merely the exemplified polyepitopes.

61. I declare that all statements made of my knowledge are true and all statements made on the information are believed to be true; and, further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereupon.

Date: 19th November 2002.

JOHN COOPER COX

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Suhrbier, et al.	Group Art Unit: 1644
Serial No.: 09/576,101	Examiner: Huynh, P. N.
Filed: May, 22 2000	Atty. Dkt. No.: FBRC:004/TMB
For: POLYEPITOPE VACCINES	

DECLARATION OF JOHN COOPER COX

EXHIBIT JCC1: *Curriculum vitae* **of John Cooper COX**

CURRICULUM VITAE

NAME: John Cooper COX

ADDRESS: 140 Dunns Road
Bullengarook Vic 3437

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Business

DATE OF BIRTH: 29th February, 1944

PLACE OF BIRTH: Geelong, Victoria

NATIONALITY: Australian

QUALIFICATIONS:

1962 - 1964	Melbourne University BSc (Chemistry & Biochemistry majors; Bacteriology sub-major)
1970 - 1971	Department of Pathology & Immunology, Monash University Medical School MSc
1966	Melbourne University Theory of Statistics
1978	CDC Atlanta, USA Laboratory methods in the detection of rabies

EMPLOYMENT:

Present	Consultant Scientist 140 Dunns Rd Bullengarook, Vic 3437 Australia
1998 - 1999	Principal Scientist, CSL Limited
1992 - 1998	Head, Immunology Research Group
1990 - 1991	Project Leader - Pertussis Project Team

Project Manager - Immunocastration Vaccines
Project Manager - Vaccines Delivery

1987 - 1989	Head, Immunodiagnostics
1986	Head, AMRAD Project Team
1984 - 1986	Head, AIDS Project Team
1983 - 1986	Head, Bacteriology R&D
1974 - 1983	Senior Scientist, Immunology Research
1972 - 1974	Scientist, Process Development
1967 - 1969	Scientist, Bacteriology Diagnostic Production Group.
1965 - 1967	Scientist, Virology Research Group

PATENTS

1. **Title:** "Improved Immunoassay"
   Inventor: John Cox
   Priority Date: 12th December 1988
   Patent No: AU624200   NZ231727   EP0485377

2. **Title:** "Recombinant Immunocastration Vaccine"
   Inventors: J. Cox, S. Edwards
   Priority Date: 29th April 1991
   Patent No: AU634379

3. **Title:** "Vaccine Preparations"
   Inventors: J. Cox, R. Sparks, N. Mason, I. Jacobs
   Priority Date: 8th January, 1993
   Patent No: AU667003   US5902565

4. **Title:** "Saponin Preparations and use thereof in Iscoms™"
   Inventors: J.Cox, A.Coulter, B.Morein, K.Lougren-Bengtsson, B.Sundquist
   Priority Date: 12th October, 1994
   Patent No: AU686891,   NZ293882

5. **Title:** "Variants of Human Papilloma Virus Antigens"
   Inventors: J. Cox, S. Edwards, E. Webb, I. Fraser
   Priority Date: 20th December, 1994
   Patent No: AU693627   US6004557

6. **Title:** "Papillomavirus Polyprotein Constructs"
   Inventors: E.A. Webb, M.B. Margetts, J.C. Cox, I. Fraser, N.A.J. McMillan, M.P. Williams, M.B.H. Moloney, S.J. Edwards
   Patent No: AU699547   NZ313108

7. **Title:** "Ganglioside Immunostimulating Complexes and Uses Thereof"
   Inventors: J.C.Cox, B.J.L.Ronnberg and S.Sjölander.
   Application No: PCT/AU/98/00453

8. **Title:** Immunogenic composition and methods related thereto
   Inventors: JC Cox, D Drane, B Moloney, A Suhrbier
   Priority date: 17th February 1999

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Suhrbier, et al.

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

DECLARATION OF JOHN COOPER COX

EXHIBIT JCC2: Amended claims

14.   A polynucleotide comprising a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) epitopes wherein each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope and wherein at least two of the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence that does not comprise a methionine.

15.   The polynucleotide of claim 14, wherein the CTL epitopes are contiguous.

16.   The polynucleotide of claim 14, wherein said polynucleotide encodes at least three CTL epitopes.

17.   The polynucleotide of claim 14, wherein said polynucleotide encodes four CTL epitopes.

18.   The polynucleotide of claim 14, wherein said polynucleotide encodes nine CTL epitopes.

19.   The polynucleotide of claim 14, wherein said polynucleotide encodes ten CTL epitopes.

20.   A vector comprising the polynucleotide of claim 14.

21.   The vector of claim 20, wherein said vector is selected from the group consisting of a viral vector and a virus-like particle (VLP).

22.   The vector of claim 21, wherein said viral vector is a vaccinia vector.

23.   The vector of claim 21, wherein said viral vector is an avipox virus vector.

24.   The vector of claim 21, wherein said vector is a VLP.

25.   The polynucleotide of claim 14, wherein at least one of said CTL epitopes is derived from a pathogen.

26.   The polynucleotide of claim 14, wherein said polynucleotide comprises a nucleic acid sequence encoding CTL epitopes derived from a plurality of pathogens.

27.   The polynucleotide of claim 25, wherein said pathogen is selected from the group consisting of Epstein Barr Virus, Influenza Virus, Cytomegalovirus, Adenovirus and HIV.

28.   The polynucleotide of claim 14, wherein at least one of said epitopes is derived from a tumor protein.

29.   The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a T helper cell epitope, a B cell epitope, or a toxin.

30.   The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a T helper cell epitope.

31. The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a B cell epitope.

32. The polynucleotide of claim 14, further comprising a nucleic acid sequence encoding a toxin.

33. A nucleic acid vaccine comprising a polynucleotide comprising:

   (i)   a nucleic acid sequence encoding a plurality of cytotoxic T lymphocyte (CTL) epitopes wherein each CTL epitope is substantially free of peptide sequences naturally found to flank that CTL epitope and wherein at least two of the plurality of CTL epitopes are contiguous or spaced apart by an intervening sequence that does not comprise a methionine; and

   (ii)   an acceptable carrier.

34. The nucleic acid vaccine of claim 33 wherein the CTL epitopes are contiguous

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
    Suhrbier, et al.

Serial No.: 09/576,101

Filed: May, 22 2000

For: POLYEPITOPE VACCINES

Group Art Unit: 1644

Examiner: Huynh, P. N.

Atty. Dkt. No.: FBRC:004/TMB

DECLARATION OF JOHN COOPER COX

EXHIBIT JCC3:

Rammensee *et al., Ann. Rev Immunol. 11*, 213-244, 1993

25226358.1

PEPTIDES NATURALLY PRESENTED BY MHC CLASS I MOLECULES

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Abstract

MHC class I molecules are peptide receptors of stringent specificity which however still allow millions of different ligands. This is achieved by the following specificity characteristics summarized as allele-specific peptide motif: Peptides are of defined length, depending on the class I allele (either 8 or 9 residues; exceptions have been observed). Typically, 2 of the 8 or 9 positions are anchors that can only be occupied by a single amino acid residue, or by residues with closely related side chains. Location and characteristics of anchors vary with class I alleles. The C terminus of the peptide ligands is frequently an aliphatic or charged residue. Such allele-specific class I peptide ligand motifs, known so far for H-2K^d, K^b, K^k, K^{k1}, D^b, HLA-A*0201, A*0205, and B*2705, are useful to predict natural T cell epitopes. The latter can be determined by extraction from cells recognized by the T cell of interest. It is not known how the class I ligands are produced in the cell, although speculative models exist. The peptide specificity of class I molecules and experimental evidence indicate that T cells are tolerant to only a small fraction of the expressed genomic sequences and are not tolerant to the remainder. The function of class I molecules is to present a collection of self-peptide samples at the cell surface for surveillance by T cells.

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INTRODUCTION

Major histocompatibility complex (MHC) molecules are peptide receptors. Cell surface MHC class I molecules present peptides derived from cellular proteins to T cells. In the normal situation, all peptides are from normal cellular proteins, and the T cells are tolerant to these normal self-peptides.

In pathological situations, for example, if a cell has been infected by a virus, foreign peptides are presented in addition to the self-peptides. The foreign peptides can now be recognized by T cells, which may destroy these cells. Thus, the system of peptide presentation by MHC class I molecules and the screening of these peptides by T cells provide the immune system with a measure to survey the interior of cells for expression of abnormal proteins and to destroy such aberrant cells. This system is instrumental in protecting the body against viral disease, or against certain other infectious agents with cytosolic parasitism, like Listeria or Plasmodium. In addition, certain tumor cells expressing peptides unfamiliar to T cells may also be eliminated. The purpose of this review is to discuss the nature of peptides presented by MHC class I molecules, the rules for peptide presentation, the ways the peptides might be processed from cellular proteins, and the significance of peptide presentation for immunity as well as for self-tolerance.

STRUCTURE OF CLASS I MOLECULES

MHC class I molecules are glycoproteins consisting of a heavy chain, about 350 amino acids, and a light chain also known as β_2-microglobulin, about 100 amino acids (1). The human MHC is called HLA; that of the mouse H-2. Class I heavy chains as well as most other MHC genes are encoded on chromosome 6 in humans and on chromosome 17 of the mouse. The heavy chain consists of three extracellular domains, α_1, α_2 and α_3, a transmembrane region, and a cytoplasmatic tail. β_2-microglobulin is noncovalently attached to the α_3 domain. The α_1 and α_2 domains form a peptide binding groove made up of a β-pleated sheet and two alpha helices, as has been revealed by X-ray analysis of HLA-A2, Aw68, and B27 crystals (2-5). The extension of the peptide-accommodating groove is about 1 × 2.5 nm, and it holds peptides in roughly extended conformation of about 8 to 10 amino acids (5-7). The peptides are tightly bound in the groove; both N and C termini are buried and are interacting with MHC residues. In addition, certain side chains of peptide amino acid residues interact with corresponding MHC residues forming pockets, whose location and shape varies with the allelic forms of the molecule. The extreme polymorphism of MHC class I genes—both mice and humans

have a couple of dozen if not hundreds of alleles at each of the three class I loci, and one allele may differ from the other by 40 amino acids (1)—i reflected in different peptide-accommodating specificities of the respectiv grooves (2-7).

APPROACHES TO ANALYSIS OF CLASS I-ASSOCIATED PEPTIDES

The classical experiments showing that virus-specific T cells recognize viral antigen in the context of MHC class I molecules were those of Zinkernagel & Doherty (8). Such T cells could also be stimulated with synthetic peptide (9), and Townsend and colleagues localized these antigens on fragment of intracellular viral proteins, for which synthetic peptides bound to unin fected cells can also be substituted (10,11). Isolation of naturally processed peptides recognized by class I-restricted T cells was not reported until 1990 (12). Two principle methods are used to isolate such peptides.

Acid Extraction of Peptides from Whole Cells

If cells are mechanically destroyed and treated with trifluoroacetic acid the majority of material precipitates, whereas small peptides including class I ligands stay in the supernatant. The heterogeneous mixture of peptides and other molecules is then separated by gel filtration and HPLC, and the individual fractions are tested for recognition by T cells (12). This is done by incubating ^{51}Cr labeled target cells expressing the relevant MHC molecule, but not the relevant antigen, with the individual HPLC fractions. This process is followed by incubation with cytotoxic T cells specific for an antigen expressed by the cells used to prepare the extract. Such an approach is useful for isolation and characterization of peptides for which specific T cells are on hand. The first naturally processed T cell epitopes have been isolated this way; some of these were identified by biochemical comparison with synthetic peptides (12-14). An advantage of this approach is that it detects not only peptides that had been bound to MHC molecules in the intact cells, but also all other peptides that have the capacity to bind to MHC molecules and are recognized by T cells (15,16).

Acid Extraction of Peptides from Purified MHC Molecules

Isolation of MHC class II molecules by immunoprecipitation followed by acid extraction of associated peptides has been reported by Demotz and colleagues (17). The first report on peptides isolated from purified class I molecules was by Van Bleek & Nathenson (18). The approach is to lyse cells with detergent, to precipitate MHC molecules by antibodies bound to a solid phase, and to dissociate peptides from MHC molecules by acid

treatment. Dissociated peptides are then separated by HPLC and can be analyzed by T cells, as with the previous approach. This led to identification of a natural vesicular stomatitis virus (VSV) epitope (18). Such MHC-eluted peptides are more readily accessible for sequencing due to less contamination with material not associated with MHC molecules. Partial sequence information of a class I-associated viral epitope has been derived by biosynthetic labeling of VSV-infected cells with radioactive amino acids Tyr and Leu; thus, the position of these residues but not of other residues was determined by this sequencing approach, confirming the identity of the VSV epitope (18). Direct sequencing of peptides, by Edman degradation and/or by mass spectrometry, allowed determination of complete ligand sequences of unknown protein origin (19–21).

THE ANTIGENIC SYSTEMS USED TO CHARACTERIZE NATURAL PEPTIDES RECOGNIZED BY CLASS I-RESTRICTED T CELLS

Peptides Derived from Foreign Proteins Expressed from Transfected Genes

Mouse cells expressing foreign proteins from transfected genes, like HLA, β-galactosidase, or ovalbumin, readily induce class I-restricted CTL in syngeneic mice and are recognized by such T cells (e.g. 22–25). The peptides recognized can be narrowed down using synthetic peptides or fragmented proteins, similarly as for virus-specific CTL. The natural peptide from β-galactosidase, as recognized by L^d-restricted CTL, has been isolated from β-galactosidase-transfected tumor cells (12).

Similarly, the natural peptide recognized by K^b-restricted CTL specific for ovalbumin was isolated, this time using purified K^b-molecules from transfected cells as source of peptide (26).

Minor Histocompatibility Antigens

Grafts exchanged between MHC-matched individuals can still be rejected, if other genes are different. Such non-MHC gene products leading to graft rejection are called minor histocompatibility antigens (27). Numerous genes of the sort are scattered throughout the mouse genome, many of which have been mapped and numbered: H-1, H-3, H-4, and so on. These antigens are recognized by MHC-restricted T cells (28). Minor H genes are also known in humans (29). The general idea is that such antigens are peptides derived from normal but polymorphic cellular proteins presented by MHC molecules, as first proposed by Townsend (10, 30, 31). Only few minor H genes have been identified so far; almost all are rather exceptional.

One is β_2-microglobulin whose allelic forms can be recognized by CTL (32–34), probably by conformational determinants, and not by recognition of β_2-microglobulin-derived peptide sitting in the MHC groove. Others are different allelic forms of a mitochondrially encoded gene (ND1, a subunit of NADH-dehydrogenase), peptides of which are presented by an odd class I molecule, Hmt, that is encoded telomeric of the H-2 region (35). (In addition, retroviral gene products can behave as minor H antigens—35a). Of those classical minor H gene products encoded autosomally and presented as peptide by classical MHC molecules, only one has been identified so far—the myxovirus resistance protein Mx recognized by K^b-restricted T cells (36). However, an additional one has been isolated as a protein (37), and several others as natural MHC class I ligands, like the K^k-restricted H-4^b antigen, the Y-chromosome encoded H-Y antigen (D^b-K^k-restricted H-4^b antigen) as well as an HLA-B35-restricted human minor H antigen (12, 15, 38).

Peptides Recognized by Alloreactive CTL

The trait leading to discovery of MHC genes was the strong rejection of MHC incompatible grafts in mice (caused by T cells) (1, 39). This reaction has its in vitro correlate in the strong activation of T cells confronted with stimulator cells expressing MHC. The specificity of such "alloreactive" T cells is rather heterogeneous. Some of such T cells might be peptide-independent, whereas a portion is clearly peptide-specific or at least peptide-dependent (16, 40–51). The analysis of naturally processed peptides recognized by several alloreactive CTL lines nominally directed against the H–2K^b-molecule has provided some insight into general aspects of protein processing in the class I pathway (16), a point discussed later. In addition, these experiments explained the high frequency of alloreactive T cells against any given foreign MHC molecule by the high number of combinatorial foreign MHC/foreign peptide complexes on any given foreign cell (16, 52, 53). The term "foreign peptide" in this context deserves closer consideration, a point discussed in the paragraph on self-tolerance.

Viral Peptides

The first naturally processed T cell epitopes to be identified were of viral origin. Identification was possible because the antigen recognized by the respective CTL had been narrowed down by transfection of individual viral genes, or truncated genes, and by analysis of CTL using synthetic peptides according to viral sequences, as pioneered by Townsend and colleagues (10, 11). The D^b-restricted influenza nucleoprotein nonapeptide ASNENMETM (Nucleoprotein 366–374) as well as the K^d-restricted nonapeptide, TYQRTRALV (NP 147–156), were identi-

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fied as natural T cell epitopes (13, 14) by comparison with longer synthetic peptide preparations known to contain the respective epitopes (11, 54). At the same time, van Bleek and Nathenson identified a K^b-restricted octapeptide of VSV nucleocapsid protein, RQVYVQGL, by eluting the natural peptide from purified K^b molecules (18). Only one additional natural viral epitope, the L^d-restricted nonapeptide YPHFMPTNL of mouse cytomegalovirus (MCMV) (55), has been identified to date (June 1992), although for many other cases likely candidates are known.

An important subsidiary point of the abovementioned work on influenza peptides was that the biological effects of a given synthetic peptide preparation may be due mainly to byproducts occurring in minute amounts. Crude synthetic IASNENMETM/ESSTLE (influenza nucleoprotein 365–380), for example, contained a very small amount of ASNENMETM that did not give an OD 220 signal in the HPLC profile and still was the most active fraction recognized by D^b-restricted, influenza-specific CTL (13, 14). This is due to the extremely high biological activity of natural epitopes, detectable in CTL assays down to the femtomolar range. This point was later confirmed with different peptide preparations and assays (56).

Peptides Recognized by Tumor-Specific CTL

Certain tumors in the mouse as well as in humans can be efficiently recognized by tumor-specific or, at least, tumor-directed CTL. Several genes coding for antigens recognized in such situations have been identified by the pioneering work of T. Boon and colleagues, for example, the antigens recognized on P815 mastocytoma cells of DBA/2, or of variants of these cells (57–60). Similarly, the antigen recognized by human melanoma-specific T cells has been identified (61). Although in these cases the genes as well as the approximate location of the T cell epitopes is known, the natural MHC ligand recognized by tumor-specific CTL was identified in only one case so far. The K^d restricted nonapeptide KYQAVTTTL is recognized by CTL specific for an immunogenic variant of P815 tumor cells (62).

THE RULES FOR PEPTIDES PRESENTED BY CLASS I MOLECULES

Pool Sequencing of Peptide Gemischs Eluted from Class I Molecules

The natural K^d-ligand TYQRTRALV (derived from influenza nucleoprotein), mentioned above, contains a Tyr (Y) residue, similar to several synthetic peptides known to contain K^d-restricted T cell epitopes and known to bind to K^d. Tyr is important for K^d-binding, as well as a Leu or

Ala residue 8 positions apart, as shown by J. Maryanski and coworkers (63). Alignment of the natural nonamer with those synthetic peptide sequences according to their Tyr residues suggested that all natural K^d ligands might be nonamers with Tyr at position 2, and an aliphatic residue like Ile, Leu, or Val, at the C-terminus (14). This hypothesis was essentially confirmed when the natural K^d-ligands isolated from P815 tumor cells were sequenced as a pool (19; Table 1). Similar approaches were also useful for other class I molecules, like K^b, D^b, and HLA-A2 (19). The results indicated that each MHC class I allelic product has its own peptide specificity, a peptide motif, characterized by an allele-specific length of 9 (K^b, D^b, A2), or 8 (K^b) amino acid residues. Certain positions, like position 2 of K^d-ligands, are occupied by residues with similar side chains, such as similar auxiliary anchors. Positions frequently but not always occupied by similar residues are called auxiliary anchors. The characteristics of the ligands of a given MHC allelic product are summarized as peptide motifs (19). The peptide motifs known so far (mostly determined by pool sequencing) are those of H-2K^d, K^b, D^b, K^k, K^kml, HLA-A*0201, A*0205, and B*2705 and are compiled in Tables 1 through 6.

Comparing these peptide motifs with synthetic peptides containing T cell epitopes suggested that most synthetic peptide epitopes determined so far went longer than the respective natural ligand. In addition, such comparison suggested in some cases that the allele-specific peptide length is not so much determined by the number of amino acid residues, e.g. 9 for D^b-ligands, but rather by the spatial length of peptides as mounted in the MHC groove, since, especially for some Pro- and Gly-containing T cell epitopes, the aliphatic C-terminus could not be aligned at position 9 but rather at position 10 or 11 (Example: SGPSNTPPEI of Adenovirus). It was suggested that certain residues like Pro (inducing kinks in peptide stretches) or combinations of Pro and Gly might contract the spatial lengths of peptides (19). Although natural class I ligands with more amino acids than the nominal allele-specific residue number have not been determined yet, optimal binding as well as T cell recognition of a Pro-containing D^b-restricted epitope with 10 instead of 9 residues (64) and of a K^b-restricted peptide with 9 instead of 8 residues (55a, 56, 65) suggested that the above assumption was correct. The notion was indeed proven recently by analysis of monopeptidic class I crystals (65a,b).

Sequencing of Individual Peptides Eluted from Purified MHC Molecules

Information on the ligand specificity of individual MHC molecules can also be derived by comparing a set of individual peptides eluted from the

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Table 1 K^d-motif (Ref. 19)

	Position[a] 1 2 3 4 5 6 7 8 9	Source	Ref.
Anchor residues	Y · · · · · · · L/J		
Frequent residues	N P M K T · · I/F/M · L		
Also observed	K F A A V B P H V A M E M I N E R V B D B D K B R D I Y E V V S A L V Q F T F N G R B R Q E R T L R L G T T K E N Y M L		
Natural ligands	T Y Q R T R A L V	influenza nucleoprotein	14,54
	S Y F P E I T H I	Protein tyrosin kinase JAK1	18,66
	K Y Q A V T T T L	Tumor antigen of P815	112
	G Y K D G N E Y I	Listeriolysin (L. monocytogenes)	73
Likely ligands[b]	R Y L E N G K E T L	HLA-A*24	19,123
	R Y L K G K E T L	HLA-Cw3	18,22
	S Y I P S A E K I	Plasmodium circumsporozoite protein	19,80,124
	S Y V P S A E Q I	Plasmodium circumsporozoite protein	19,80,123

[a] Anchor positions in bold.

[b] Sequences that (i) fit to the motif and (ii) are contained in synthetic peptides recognized by the respective T cells, or correspond to the synthetic peptide with highest biological activity.

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Table 2 D^b motif (Ref. 19)

	Position 1 2 3 4 5 6 7 8 9	Source	Ref.
Anchor residues	· · · · N · · · M		
Frequent residues	M I K L L E F P Q V V		
Also observed	A A G D A D F L N Q N T Y E H I D T Q K F G V V S P M E Y S E Y T T Q Y H I K P S		
Natural ligand	A S N E N M E T M	influenza nucleoprotein	11,13
Likely ligands	S G P S N T P P E I	Adenovirus	19,64
	B G V E N P G G Y C L	LCMV	19,120
	S A I N N V A Q K L	SV40	127
	C K G V N E E Y L	SV40	122
	Q G I N N L D N L	SV40	127
	F Q P Q N G Q F I	LCMV	128
	A S N E N M D A M	FLU NP 1968	75

grooves. This approach has the advantage that the protein of origin can be tracked down. The first natural MHC ligand to be directly sequenced was SYFPEITHI (19), a prominent self-peptide occupying about 5% of K^d molecules on P815 tumor cells. A matching peptide stretch, SFFPEITHI, was found in a human sequence, protein tyrosine kinase JAK 1 (56). Thus, the natural K^d ligand suggested that the corresponding mouse JAK 1 sequence was SYFPEITHI, a notion confirmed in the meantime by sequencing of the mouse gene (A. G. Harpur, A. Ziemiecki,

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Table 3 Kᵇ-motif (Ref. 19)

	Position[a]								Source	Ref.
	1	2	3	4	5	6	7	8		
Anchor residues					F			L		
						Y				
Frequent residues			Y					M		
Also observed	R		N		R		T	N I		
	I		Q		I		I	Q V		
	L		E		E			E K		
	S		X		B			B G		
	A		T		Q			Q		
	H		Y		P			P		
			H							
Natural ligands	R	G	Y	V	Y	Q	G	L	VSV	18
	S	I	N	F	E	K	L		Chicken ovalbumin	28
	H	I	Y	E	F	P	Q	L	Gag protein of PB15	134
Likely ligands	F	A	P	G	N	Y	P	A L	Sendai virus	55
	S	I	I	E	F	A	R	L	HSV-1 glycoprotein B	H. Rosenthal, pers. communication

[a] Auxiliary anchor position underlined.

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Table 4a Kᵈ-motif[a]

	Position								9	Source	Ref.
	1	2	3	4	5	6	7	8[b]			
Anchor residues		E							I		
Frequent residues		K									
		N									
		Y									
		M									
Also observed	V		Q	L	A	N	T				
	F		I	Q	K						
			L	P	H						
			F	T							
			P	V							
			H	F							
			T	B							
Likely ligands	F	E	G	T	G	N	L	I		Influenza Haemagglutinin 2	128
	F	E	A	N	G	N	L	I		Influenza Haemagglutinin 1	123
	I	E	G	G	W	T	G	M I		Influenza Haemagglutinin 2	123
	G	E	F	L	L	E	K	R I		SV 40 T antigen	128
	Y	E	N	D	I	E	K	K I		Plasmodium circumsporozoite protein	129
	S	D	Y	E	G	R	L	I		Influenza Nucleoprotein	129
	D	E	L	D	Y	E	N	D I		Plasmodium circumsporozoite protein	129

[a] H. Norda, K. Falk, O. Rötzschke, S. Stevanovic, G. Jung, and H.-G. Rammensee, submitted.
[b] The C-terminal residue appears to be invariably Ile; the C-terminus can be at far wider as at 9 although the majority of natural ligands are probably octamers.

A. F. Wilks, K. Falk, O. Rötzschke, H.-G. Rammensee, unpublished observation).

The approach of directly sequencing individual MHC ligands has been followed up by several laboratories for both class II (67-69) and class I (20, 21, 70). The peptide motif of HLA-B*2705 has been determined this way by analyzing 11 complete or partial sequences of normal B27 ligands (20). The technique was greatly improved by using mass spectrometry for microsequencing, yielding complete or partial sequences of some 9 HLA-A2 ligands (21), that entirely confirmed the A2 motif as determined by genissch sequencing. The natural ligands of the different class I allelic

products—detected by direct sequencing or by comparison with synthetic peptides—are also compiled in tables 1 through 6 as well as some likely natural ligands. In addition, two naturally processed ligands have been described for L⁴-molecules, YPHFMPTNL and LSPFPFDL (51, 55). Together with partial information on the L⁴-specificity from a pool-sequencing attempt (19), and with information on optimal synthetic T cell epitopes (59, 71) the L⁴ motif is likely to be XPXXXXXXL, with other aliphatic residues allowed at 9, and Ser tolerated at 2.

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Table 4b Kᵇᵐᵈ-motif[a]

Position	1	2	3	4	5	6	7	8
Anchor residues								I
Frequent residues					E			K
Also observed					Q	H	P	A	B
					G	Q		R	Y
					P	G		M	
								M	
								P	
								Y	

[a]M. Norda, K. Falk, O. Rötzschke, S. Stevanović, G. Jung, and H.-G. Rammensee, submitted.

A method slightly at variance to direct sequencing of MHC ligands is to label cells metabolically with individual radioactive amino acids before peptide elution and to determine the relative position of that residue within the ligand. This approach has been used for identification of a natural VSV epitope (18), to confirm frequent usage of Tyr at positions 3 and 5 and Leu at 8 of the Kᵇ-motif and to determine the position of these residues in several natural ligands of Kᵇᵐᵈ and Kᵇᵐᵈ molecules (72).

Prediction of Natural T Cell Epitopes Using the Motifs

If the sequence of a cellular protein of immunological interest is known, comparing the sequence with the allele-specific peptide motif of a relevant MHC molecule indicates which stretches of the protein are likely to be natural MHC ligands. An example is chicken ovalbumin expressed in H-2ᵇ mouse cells. The ovalbumin epitope recognized by Kᵇ-restricted T cells had already been narrowed down to one contained in the sequence IINFEKL TEWTSSNVNEER (chicken ovalbumin 258–276) (24, 73). The Kᵇ-restricted peptide motif requires an octamer with an aromatic residue at position 5 and an aliphatic one at 8 (19). Since the only aromatic residue in the above sequence is the Phe (F) at position 4, an additional residue at the N-terminus, Ser, was required by the motif, to result in the octamer SIINFEKL. This prediction was experimentally confirmed by elution of the natural ovalbumin peptide from Kᵇ molecules, followed by biochemical comparison to synthetic SIINFEKL (26). Similarly, the Kᵈ-restricted pep-

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Table 5r HLA-A2.1-motif (A*0201) (Refs. 19, 21)

Position	1	2	3	4	5	6	7	8	9	Source	Ref.
Anchor residues		L							V		
Frequent residues		M			E		V		K		
		K									
Also observed	A	G	I	I	A	E	L				
	L	P	K	L	Y	I					
	F	F	Q	Y	Y	H	D				
	K	P	T	N	Q	V	Q				
	M	N	S	Q	A	A					
	Y	S	V	F	P	H					
	V	R	V								
	B	D	H								
	G	V	A								
	T	L	A								
		H	R								
		W	Q								
Natural ligands[a]	S X P S Q G X Q V										
	L L D V P T A A V .									Human IP 30 signal peptide	88
	L L L D V P T A A V Q A									Human IP 30 signal peptide b	88
	L L L D V P T A A V									Human IP 30 signal peptide	96
	G X V P F K X V 8 V									P61 myc... protein-phosphatase 2A	88
	S L L P A I V E L										88
	B X X V R A X E V										88
	K X N E P V X X X										87
	Y L L P A I V H I										88
	T L W V D P Y E V										88
Likely ligands	I L K E P V H G V									HIV reverse transcriptase	79
	G I L G F V F T L									Influenza matrix protein	19, 130, 133
	I L G F V F T L T V									Influenza matrix protein	19
	K I G E F Y N Q M									Influenza B1P	131

[a]X: Leu or Ile.

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Table 5b HLA-A*0205-motif (Ref. 135)

	Position								
	1	2	3	4	5	6	7	8	9
Anchor residues									L
Frequent residues		V	Y	G	V	I			K
		L	P	E	Y				
		I	F	D	L				
		Q	I	I					
Also observed		M	K		V	Q			
		H			T				
					L				
					A				
					R				

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Table 6 HLA-B27-motif (Ref. 20)

	Position									Source	Ref.
	1	2	3	4	5	6	7	8	9		
Anchor residues		R									
Also observed	R	Y	Q	K	R	T	E	K			
	Q	I	K	E	I	V	K	R			
	K	V	L	P	V	G	V	Y			
	A	W	D	G	A	I	L	F			
		B	N	R	L	E	H	L			
		I	E	P	R	Q					
		F	T	A							
		L	P								
Natural ligands	R R Y Q K S T E L									Human Histone H3, H3.3	20
	R R I K E I V K K									Human Hsp 89α	20
	R R V K E V V K K									Human Hsp 89β	20
	R R W L P A G d a									Human elongation factor 2	20
	R R S K E I T V R									Human RNA-dependent RNA-helicase	20
	G R I D K P I L K									Ribosomal protein	20
	f R Y N G L I H I									Rat G3 S ribosomal protein L28	20
	K A F E G L T Q R										20
	R A F T R P E H -										20
	R R I S G V D R Y										20
	A R L F G I R A K										20
Likely ligands	S R Y W A I R A K									Influenza nucleoprotein	132
	S R Y W A I R T R									Influenza nucleoprotein	20, 132
	K R W I I L G L N K									HIV gag p24	20, 135
	G R A F V T I G K									HIV gp 120	20

*Compiled from the single peptides shown below.
*Lower case letters: residues of lower confidence.

tide recognized by CTL specific for a variant of P815 tumor cells was predicted and demonstrated to be KYQAVTTTL (52). In the case of a Listeria protein, Listeriolysin, the epitope recognized by Kᵈ-restricted CTL had not been narrowed down before by synthetic peptides or truncated genes. The Kᵈ-restricted peptide motif indicated 8 nonamers in the Listeriolysin sequence as candidates for Kᵈ-ligands, one of which, GYKDGNEYI, was indeed the correct one (73a). Thus, the MHC allele specific peptide motifs are useful for identifying natural T cell epitopes in proteins of immunological interest.

Allele-specific Peptide Motifs Are Not Just "Binding Motifs"

The majority of peptides that have been described so far to bind to class I molecules do not adhere to the respective peptide motifs (e.g. 14, 63–65, 74–78a). This reflects the capacity of class I molecules to bind peptides of between 4 and 28 amino acids, no matter whether they do or do not contain the relevant motif. However, the binding of the natural ligands is in most cases several orders of magnitude better than binding of the "wrong" peptides (13, 14, 56, 79). Because the peptide motifs were determined not by screening for peptides that are the best binders but rather by analyzing the MHC ligands occurring naturally, these motifs incorporate each single step required for a peptide to become a natural MHC ligand. One of these steps, of course, is binding; others, however, include the processing of peptides that must be influenced by the specificities of the enzymes involved, and probably also the transport of either proteins

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or peptides between cellular compartments (see below). It is, therefore, scientifically not exact, and sometimes rather misleading, to talk of allele specific peptide motifs as "binding motifs". In the case of K^d, on the other hand, a true peptide binding motif has been established by Maryanski and coworkers, and it has the same basic characteristics as the motif based on natural ligands (80). Thus, peptide binding motifs and natural ligand motifs of MHC molecules may coincide but should not be expected to do so a priori.

Peptide Specificity of "Nonclassical" Class I Molecules

In addition to the "classical" class I genes, HLA-A, B, C, and H-2K, D, L, many other class I genes are encoded on the respective chromosome; some of them are expressed on the cell surface (1). Some of those "non-classical" class I molecules have been shown to present antigen in a few cases (e.g. 15, 81, 82). For the Hmt molecule, which presents a mito-chondrially encoded minor H antigen, Mta, to CD8+ T cells (35), studies with synthetic peptides have indicated that this molecule is able to bind peptides formylated at the N-terminus (83). Although natural Hmt-ligands have not been identified yet, these data indicate that Hmt molecules are specialized in presenting formylated peptides that might occur as fragments of proteins from prokaryotes or mitochondria (83). Analysis of natural ligands of another nonconventional class I molecule, glycophospholipid-anchored Qa-2, indicated a peptide motif similar to that of ordinary class I molecules, except that it seemed to be much more stringent in its specificity. According to this study, Qa-2 ligands are nonapeptides with two anchors (His at 7 and L, I, or F at 9) and four auxiliary anchors (O. Rötzschke, K. Falk, S. Stevanović, B. Grahovac, M. Soloski, G. Jung, and H.-G. Rammensee, submitted); thus, only a relatively small number of nonapeptides would fit to Qa-2. It appears, therefore, that at least some of the nonclassical class I molecules, like Hmt and Qa-2, are peptide receptors of narrower specificity than ordinary class I molecules.

HOW DO CELLS MAKE THE PEPTIDES?

This question can be answered by a plain "not known" (June 1992). However, there is a long list of observations that give hints towards the mechanisms involved. Consideration of some of these observations, and disregard of others, has led to a view now widely accepted although unproven.

The Common Dogma

A widely publicized view of the mechanism for peptide loading of class I molecules is the following (see 84–101). Proteins are cut into the correct

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8-mers or 9-mers by proteasomes in the cytosol. The resulting peptides are then transported to the ER by ATP-dependant peptide transporters encoded by TAP 1 and TAP 2 genes located in the neighborhood of MHC class II genes. In the ER, class I molecules select the peptides fitting to the respective motifs and bring them to the cell surface. There is no doubt on the last step, i.e. that class I molecules transport peptides to the plasma membrane. There is also no doubt that TAP 1 and TAP 2 genes are essential for proper function of class I molecules (88, 94); indeed, the TAP gene products influence the peptide pattern that could be eluted from a rat class 1 molecule (100). The other parts of the dogma, including the compartmentalization, are merely speculative. There is no evidence so far that "peptide transporters" really do transport peptides, nor is there an indication on the identity of peptides potentially involved, although a good dozen of papers or editorials have been published in Nature or Science so far on those genes or molecules. Another explanation (although not very likely) is that the latter act as MHC-flippase, that is to toggle class I molecules from the ER lumen to the cytosol and back, as has been acknowl-edged in a recent review (99). The following paragraphs deal with obser-vations that do not easily fit with the above model.

Protein But Not Final Size Peptide Is Found in the Cytosol

One of the first naturally processed T cell epitopes to be isolated (but not identified) was derived from β-galactosidase expressed from a transfected leaderless gene in P815 tumor cells (12). The transfectant, P13.1, contains high amounts of β-galactosidase in the cytosol, as detectable by β-galacto-sidase enzyme activity. However, the naturally processed peptide recog-nized by L^d-restricted T cells that can be found in whole cell extracts as well as in the membrane fractions, was not detected in the cytosol (12 and O. Rötzschke, K. Falk, H.-G. Rammensee, unpublished). Thus, if the final sized peptides are really produced in the cytosol, their duration or half-life in this compartment must be too short to allow detection.

Occurrence of Peptides in Whole Cell Extracts Is MHC Dependent

As mentioned above, whole cell extracts from β-galactosidase expressing P13.1 cells ($H-2^d$) do express the L^d-restricted β-galactosidase T cell epitope. EL4 tumor cells ($H-2^b$) transfected with the same gene and expressing even more β-galactosidase activity in the cytosol do not contain the L^d-restricted peptide (12 and K. Falk, O. Rötzschke, H.-G. Rammensee, unpublished). This result indicated that coexistence of the correct class I molecule is required for occurrence of a given T cell epitope, since EL4 cells do not express L^d. This notion was confirmed with several

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present in spleen cells of all mouse strains, in human cells (Jurkat), and even in yeast or earthworm cells, indicating that this peptide—obviously derived from a conserved protein—is really MHC-independent.

Considering these data, one could speculate that the final size peptides—the class I ligands—are always derived from MHC-independent larger precursor peptides, and that detection of the latter depends on the chance that a given T cell happens to recognize not only the natural class I ligand, but also its larger precursor.

An alternative explanation for the observed MHC-dependency of peptide occurrence in cells, however, would still be "determinant protection" of entirely preprocessed peptides by binding to MHC molecules (15, 104, 105). Such a mechanism, however, would pose a principal problem that is discussed in one of the following paragraphs.

The Location of a Peptide Stretch Within a Protein Does Not Influence the Identity of Processed Peptides

The sequence coding for a natural L^d ligand, YPHFMPTNL, from murine cytomegalovirus immediate early protein, was inserted into several sites of an unrelated gene, with or without linker sequences, and the construct was expressed in cells (55). In all cases, the natural L^d ligand was the same nonapeptide, although considerable quantitative differences were observed. In another study, when viral epitopes were inserted into various protein sites, the natural ligands were not identified, but recognition of all the different constructs by the same T cells suggested that the same epitope was processed in all cases (106). Thus, although the data base for these kind of experiments is still small, it appears that the identity of a processed class I ligand is generally independent of the flanking amino acid residues in the protein of origin, and that all the informations for cleavage sites must be within the ligand sequence. In still another study, certain flanking sequences of epitope-containing minigenes transfected into cells did not allow T cell recognition (107); because the identity of the corresponding ligands had not been identified, the data are still consistent with influence of flanking sequences on quantity but not identity of peptides.

Human Cells Know How to Make the Peptides for Mouse Class I Molecules

The alloreactive, K^b-specific CTL line 2STO-3 not only recognizes $H-2^b$ mouse cells, but also human Jurkat tumor cells transfected with K^b (16). A peptide recognized by this CTL line can be isolated from both mouse and human cells expressing K^b. The peptide is probably derived from a protein conserved between mouse and human. Biochemical comparison of the peptide from mouse cells with that from human cells indicates that

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other antigens, including minor H and viral antigens (15, 16, 38, 55). MHC dependency of the cellular peptide content of cells was formally proven by comparing the peptides extracted from class I wild type and mutant mice (15) and was confirmed by analyzing cells transfected with class I genes (38, 102) as well as with tissue from transgenic mice (103). A semiquantitative study, using the K^b-restricted minor H peptide, $H-4^b$, indicated that a high copy number (5000-fold over detection limit) of K^b-restricted $H-4^b$ peptide is present in an extract from 10^9 P815 cells transfected with K^b, but no detectable peptide is detected in untransfected P815 cells (102). These data suggest that, at a given point, not a single detectable copy of the final sized $H-4^b$ peptide is present in cells not expressing K^b. An exception to the strict MHC dependency of peptide occurrence in whole cell extracts has been reported recently for one L^d ligand recognized by alloreactive T cells (51).

Precursor/End Product Relationship?

The minor H antigen, $H-4^b$, mentioned above, shows a peculiarity informative for general considerations on antigen processing. In peptide mixtures eluted from purified K^b molecules of $H-4^b$ expressing cells, T cells recognize but one $H-4^b$ peptide (called $H-4^b$ main peptide) (15). In contrast, however, $H-4^b$ specific CTL recognize two peptides in whole cell extracts of K^b expressing $H-4^b$ cells (15, 102). These two peptides can be distinguished by their behavior on HPLC; one of them is identical to the $H-4^b$ main peptide, the other is called the "$H-4^b$ prepeak." If whole cell extracts of cells lacking K^b are analyzed, only one $H-4^b$ peptide is found, the $H-4^b$ prepeak. Thus, the $H-4^b$ main peptide is strictly MHC dependent, whereas like most other class I restricted peptides (see previous paragraph), the $H-4^b$ prepeak peptide (being not a natural K^b ligand) is MHC independent in its occurrence in whole cell extracts. We speculated that the two peptides may be linked by a precursor/end product relationship (15). The MHC independent one could be a precursor peptide cut out from the $H-4^b$ protein by enzymes that are independent of MHC molecules. This larger precursor—which happens to be able not only to bind to K^b but also to be recognized by K^b-restricted CTL—is then transported to the compartment of class I loading, binds to K^b, and then is trimmed to give the final sized ligand, according to our hypothesis (7, 15, 19). Alternatively, the MHC independent peptide might be derived from another proteolytic pathway unlinked to class I-restricted processing.

A similar situation—two peptides found in whole cell extracts, only one of which is MHC dependent and a natural class I ligand—was found for the peptide-specific, alloreactive, K^b-specific CTL clone 27.5 (16). In addition to an MHC-dependent peptide, this clone recognizes a peptide

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both are identical (108). Another ligand, recognized by CTL clone 9.6, yielded similar data, thus confirming the notion.

Ovalbumin-specific, K^b-restricted CTL recognize the ovalbumine peptide SIINFEKL on K^b molecules of mouse cells (26), a point mentioned previously. Human HeLa cells transfected with both the ovalbumin gene and the K^b gene are also recognized by above CTL, and SIINFEKL is a natural ligand of K^b molecules of those HeLa transfectants (O. Rötzschke, K. Falk, N. Shastri, H.-G. Rammensee, submitted).

Thus, at least in those three examples, human cells process the same peptides from a given protein, if the relevant class I molecule, K^b in the examples given, is expressed in the cells. This indicates that the mechanism cutting proteins into peptides for class I loading—in its specificity—is conserved between different species. Moreover, this specificity is also the same between different tissues, and also between different mouse strains, since minor H peptides like $B-4^b$ or H-Y appear to be the same no matter which tissue or mouse strain with correct MHC expression is analyzed (15, 103). Even tissue like brain tissue, normally almost class I negative (and thus, minor H peptide negative), can be induced to express the same H-4^b peptide as other tissue upon expression of transgenic K^b (103).

Extrapolating these data suggests that a single mouse brain cell, for example, is instantly able to provide any peptide ligand required not only by any of the hundreds of mouse class I molecules but also by the thousands of different class I molecules in humans and all other mammals. The commonly accepted model for antigen processing—postulating complete peptide processing before contact with class I molecules—would require a constant pool of ten of thousands of processed ligands fitting to all the class I molecules of all mammals, and this even in cells like brain cells that don't even express their own class I genes.

In addition to this conserved, nonpolymorphic general mechanism, however, polymorphism in peptide loading that has been shown for the class I system in rats (100) and suggested for B27 molecules in humans (109), might exist as an epiphenomenon. One possibility is also that the conserved mechanism works for all class I molecules requiring a hydrophobic C-terminus of peptide ligands, whereas the polymorphic mechanism works for class I ligands with a charged C-terminus, as for B27 ligands. (The B27 molecule appears to have a flexible pocket for the C-terminal peptide side chain able to accept either hydrophobic or charged residues; 110). Thus, the observed functional polymorphism might be due to the presence or absence of transacting factors (TAPs or proteasomes?) that are able to generate, transfer, or treat otherwise peptides terminated by charged residues.

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A Model for Peptide Processing

Some of the above observations do not easily fit into the commonly accepted model on peptide processing. We have, therefore, proposed an alternative model that would explain those observations (7, 15, 19). The main point is that it assumes an instructive role for class I molecules in processing. We hypothesize that a protein is first degraded into (precursor-) peptides larger than the final class I ligand (Figure 1). This can be, for example, in the cytosol, but is also conceivable in other compartments. The endopeptidase(s) doing the cutting could have a specificity such that cleavage occurs C-terminal of hydrophobic (Met, Ile, Leu, Val, Phe) or charged residues (a specificity covered by proteasomes); this way the precursor peptides would already have the correct C-terminus fitting to most class I motifs known so far. The resulting peptide precursors—if not produced in the ER itself, like leader peptides would be, as we had suggested (16)—would then be translocated to the site of class I loading, that is, the ER or the early Golgi. This might be done by the putative ATP-dependent peptide transporters which might be specific for the respective C-terminus or size, although there is no evidence so far that these molecules

Figure 1   Model for antigen processing in the class I pathway. For explanation, see text.

transport peptides and, at least in in vitro systems, it appears that peptides can cross the membrane of microsomes without requiring ATP (111–113). The precursor peptide would then bind with low affinity to fitting class I molecules, for example, first by contacting a conserved positive charge (Lys 146) of the groove with the peptide's C-terminus, and then by insertion of anchor residue side chains into the allele specific MHC pockets. The N-terminus, still too long, would then be trimmed to the allele-specific length by an unknown peptidase activity that could be an exopeptidase. As soon as the final size of the peptide is reached, it would snap into the groove and be bound with high affinity.

Proof for natural binding of larger peptides to A2 molecules as well as their trimming down to nonamers (from both ends, however) has been convincingly demonstrated by the work of Henderson et al, and Wei & Cresswell, who looked at natural peptides derived from a leader sequence (70, 98; Table 1). This work, in addition, confirmed our earlier hypothesis that leader peptides generated in the ER can serve as class I ligands (16).

It is clear that most elements of the alternative model described in this paragraph are highly speculative; since the model, however, can explain some of the intriguing facets of class I-restricted antigen processing, it may be useful as a working hypothesis.

HOW MANY PEPTIDES ARE PRESENTED?

Copy Number of a Given Peptide

Comparing the amount of natural VSV-derived peptide RYQVYQGL found on K^b molecules on infected cells with a defined amount of synthetic peptide, it was estimated that about 5% to 10% of K^b molecules of an infected cell are occupied with the VSV peptide (18). If we assume that 10^5 K^b molecules are expressed on a cell, this would amount to 10,000 RYQVYQGL peptide copies per cell. On the other hand, the K^d-restricted peptide TYQRTRALV of influenza is found only in 200 to 500 copies per infected cell (14). Similarly, the D^b-restricted influenza peptide ASNENMETM occurs in about 200 copies per cell (14), and the K^b-restricted ovalbumin derived peptide SIINFEKL as well as the tumor-associated K^d-ligand KYQAVTTTL is found in around 100 copies per respective cell (26, 62). The prominent self-peptide SYFPEITHI from K^d molecules of P815 tumor cells, representing the most abundant peptide species on K^d in this cell, is found in about 10,000 copies per cell (19). Thus, the copy number of individual peptides can be rather variable; judging from chromatography profiles of peptide pools eluted from class I molecules, it appears that on normal cells a few peptides are in the copy number range of SYFPEITHI, whereas most other peptides are at a much

lower rate, that is in the range of a couple of hundred copies. This fits well with mass spectroscopic analysis of A2-eluted ligands (21), and also fits well with the minimal number of synthetic peptides required to detect class I-restricted T cell recognition, if the peptides are added to the cells from outside (114, 114a).

The Number of Different Peptides per Cell

If a cell expresses 1 or 2 x 10^5 class I molecules of one kind, and the average copy number of a given peptide is 200, one would expect about 500 to 1000 different peptides per cell (14). Mass spectrometric analysis has allowed the detection of 200 different peptides eluted from A2 molecules, representing 50% of the A2-ligand population (21). Due to limits of detection, many low-copy number peptides could have been missed, and it was estimated based on the data that the number of peptides could exceed 1000. Thus it appears reasonable to assume that a given class I species presents around 1000 different peptides, with a rather broad range of copy numbers, however.

IMPLICATIONS FOR SELF-TOLERANCE

Self-Tolerance of Self-Peptides Is MHC restricted

The phrase "MHC restriction of self-tolerance" describes the events leading to the silencing of self-reactive T cell clones during their differentiation (115); they are dependent on the same kind of T cell recognition as in mature T cells, that is, recognition of MHC and antigen. Several years ago we learned that in bone marrow chimeras self minor H antigens do not induce tolerance if they are not presented with self MHC molecules, and normal mice do contain T cells reactive to self minor H antigens if presented on foreign MHC molecules (116–118). However, the antigens involved were not characterized at the molecular level, and the notion has been challenged (119). Peptide-specific alloreactive T cells allow us to readdress this question. The K^b-restricted CTL clone 27.B2, derived from a bm1 mouse (expressing K^{bm1}) recognizes a natural ligand of K^b that happens to be also a natural ligand of K^{bm1}. 27.B2 CTL recognize this peptide only if bound to K^b molecules, and not if bound to self K^{bm1} molecules (16, 120). Similar settings have been found for other alloreactive CTL (50). It can be concluded that T cells can distinguish if the same peptide ligand is presented by different class I molecules, and also that T cells in the bm1 mouse have not been negatively selected against the complex of self-peptide and foreign MHC molecule. Thus, negative selection of T cells during differentiation must require recognition of peptide ligands bound to MHC molecules.

T Cells Are Only Tolerant to Self Peptides That Are Actually Presented

Negative selection only of T cells recognizing self-peptides presented by MHC molecules would implicate that T cells are not at all tolerant to self-peptide not naturally presented on self MHC molecules. This notion was confirmed experimentally. B6 T cells could readily be stimulated with synthetic peptides according to stretches of a self-protein, β-microglobulin, to result in peptide-specific, class I–restricted CTL (78). Those peptides did not contain K^b or D^b-restricted motifs, and the CTL did not recognize $H-2^b$ cells expressing β_2-microglobulin. Thus, the respective peptides are not presented naturally by cells but can bind to $H-2^b$ molecules and induce CTL responses. Similar responses were obtained by stimulating T cells with trypsin-digested self-proteins (78). Thus, "self" for class I–restricted T cells is a rather limited selection of peptides derived from self-proteins, excluding the majority of self-protein sequences (14, 121). Consequently, peptides derived from that majority are treated as "foreign" by T cells, although derived from self sequences.

A COMPARISON WITH CLASS II

The approach of isolating and sequencing natural ligands of MHC molecules has also been applied for class II (67–69a). Differences distinguishing characteristics of class II ligands from those of class I are: (i) Class II ligands are longer, ranging roughly from about 12 to 20 residues. (ii) A given class II species can present peptides of various lengths, that is, strict allele specific length requirements are not observed. The class II peptide groove, therefore, appears to be open at both ends. (iii) Class II molecules appear to have allele-specific peptide motifs as well, although in the first publication on identified ligands, obvious motifs were not observed (67). More recent evidence indicates motifs containing two or three anchor-like positions that are not as stringent in occupancy requirements as is the case with class I molecules. A simplistic cartoon visualizing the principal differences between class I and class II ligands is in Figure 2.

CONCLUDING REMARKS

The function of MHC class I molecules is to display at the cell surface a selection of small peptides derived from cellular proteins. The selection is such that, on average, every cellular protein has a chance to participate with one or two peptides in this sampling (14, 121). Each member of a species displays her or his individual peptide selection, since the rules for

Figure 2 The principle differences between class I and class II ligands in a simplistic view.

peptide presentation are determined by the polymorphic MHC alleles (and in addition by the polymorphic *trans*-acting factors). An individual's T cell population is tolerant against his or her self-peptide collection, since all T cells recognizing one of these self-peptides are silenced during differentiation. If a new peptide appears, T cells can recognize it and attack the cell expressing it, eventually the death of the cell or necrosis of the tissue results. New peptides are likely to occur after a cell has been invaded by new genes, such as genes from viruses or other pathogens. Mutations in self-proteins could also lead to new peptides (122), although the chance that a point mutation hits a stretch carrying the motifs relevant for a cell is small. A third setting that may lead to occurrence of new class I ligands is the aberrant expression of proteins (60). In addition, the aberrant up-regulation of self-proteins could also lead to T cell recognition, since T cells can distinguish not only between different peptides but also between different quantities of the same peptide (55). Thus, peptides presented by class I molecules allow for control of the cell's interior through the immune system.

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THE IL-2 RECEPTOR COMPLEX: Its Structure, Function, and Target Genes

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Abstract

Proliferation of T lymphocytes is triggered by the interaction of IL–2 with its specific receptor following T lymphocyte activation. The receptor for IL–2 consists of at least three distinct subunits, the α chain (IL–2Rα), the β chain (IL–2Rβ), and the γ chain (IL–2Rγ). Although the role of IL–2Rγ in IL–2 signalling remains unclear, IL–2Rβ is the subunit critical for receptor-mediated signalling. Because IL–2Rβ lacks any apparent catalytic motifs, IL–2Rβ may be physically or functionally coupled to other signalling molecules.

Structure-function studies of IL–2Rβ have revealed that at least two distinct cytoplasmic regions of IL–2Rβ are involved in IL–2-induced cellular signalling. The "serine-rich" region of IL–2Rβ was identified as a region critical for IL–2-induced mitotic signalling from experiments in which IL–2Rβ mutant cDNAs lacking a particular cytoplasmic region or regions were expressed in an IL–3-dependent mouse pro-B cell line (BAF-B03). Meanwhile, another cytoplasmic region of IL–2Rβ, the "acidic" region, is responsible for its physical association with an src-family protein tyrosine kinase (PTK), p56[lck], and is critical for activating the p56[lck] PTK following IL–2 stimulation.

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The Epitopes of Influenza Nucleoprotein Recognized by Cytotoxic T Lymphocytes Can Be Defined with Short Synthetic Peptides

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Summary

A proportion of cytotoxic T lymphocytes (CTL) responding to infection by influenza recognize target cells that express the viral nucleoprotein. Recent work showed that CTL can recognize short overlapping regions of large nucleoprotein fragments expressed in transfected L cells. This led to the suggestion that CTL recognize segmental epitopes of denatured or degraded proteins in a similar way to helper T cells. One corollary of this idea is that CTL should recognize appropriate short peptides on the target cell surface. We demonstrate that the epitopes of nucleoprotein recognized by CTL in association with class I molecules of the major histocompatibility complex in both mouse and man can be defined with short synthetic peptides derived from the nucleoprotein sequence.

Introduction

Mice and humans retaliate with a vigorous cytotoxic T lymphocyte response to infection by Influenza A viruses (reviewed by Askonas et al., 1982; Townsend and McMichael, 1985). Cytotoxic T lymphocytes (CTL) generally express the Lyt2 positive (CD8 in the human) surface phenotype and recognize and kill infected target cells that share class I molecules of the major histocompatibility complex (MHC) with the host in which the CTL developed (reviewed by Swain and Dutton, 1980; Dialynas et al., 1983; Blanden et al., 1976; Zinkernagel and Doherty, 1979). CTL may play an important role in limiting the spread of virus in vivo (reviewed by McMichael et al., 1983).

Little is known about the nature of the viral epitopes recognized by CTL on the surface of an infected target cell. Recently a major population of CTL from influenza infected mice have been shown to recognize murine L cells (a fibroblast line) that express the viral nucleoprotein (NP) in isolation after DNA mediated gene transfer (Townsend et al., 1984a). Recombinant vaccinia engineered to express influenza NP also has been used to demonstrate human (McMichael et al., 1986) and murine (Yewdell et al., 1985) CTL that recognize the NP molecule. Recent evidence has shown that other nonglycosylated proteins of the virus can also be responsible for inducing cytotoxic T cells (Yewdell, personal communication).

These findings raised the question of how viral components that are not transmembrane proteins and do not have recognizable amino-terminal leader sequences are transported to the plasma membrane of the target cell, where CTL recognition is assumed to occur. To investigate this, L cells were transfected with a series of deletion mutants of the influenza NP gene in an attempt to identify sequences required either as epitopes recognized by CTL or as signals for membrane transport (Townsend et al., 1985).

Epitopes were localized by comparing L cells expressing large fragments of NP that overlapped over short regions. In this way two regions of sequence recognized by CTL were identified in a continuous segment of 59 amino acids between residues 328 and 386 of the molecule. No leader-like sequence could be located because nonoverlapping amino-terminal and carboxy-terminal fragments of NP were recognized equally well by appropriate CTL. This implied that the two ends of the molecule could be transported to the plasma membrane independently of each other. In addition, although all of the fragments of NP expressed in transfected cells could be recognized by specific CTL, some were no longer detected with antibodies that bound the complete folded molecule.

One possible explanation for these results is that nonmembrane viral proteins are degraded in the cytoplasm of the infected cell, producing short denatured peptides that are exported from the cell by some unknown mechanism. Such degraded viral proteins may then become available for recognition by CTL in association with class I MHC molecules in a way similar to that in which helper T cells recognize denatured or degraded proteins with class II molecules (reviewed by Grey and Chesnut, 1985; Unanue et al., 1984).

One of the main predictions of this proposal is that CTL should be able to recognize appropriate short synthetic peptides corresponding to linear regions of the NP sequence when they are added in vitro to the target cell surface. We describe experiments that define the epitopes of influenza nucleoprotein recognized by class I restricted CTL from both man and mouse using short synthetic peptides. Both the minimum length and the concentration required are similar to those required for recognition of protein antigens by class II restricted helper T cells. The results are consistent with the view that all somatic cells bearing class I molecules may be capable of degrading and presenting newly synthesized viral proteins to CTL.

Results

Murine and human CTL were tested for their ability to lyse ^{51}Cr-labeled target cells exposed to a variety of peptides derived from the nucleoprotein sequence (see Tables 1 and 3). Cloned murine CTL also were tested for their

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Table 1. Amino Acid Sequences of the Nucleoproteins from A/PR/8/34 and A/NT/60/68 between Residues 328 and 388

	330	340	350	360	370	380
A/NT/60/68	LVWMACNSAAFEDLRVLSFIRGTKVSPRGKLSTRGVQIASNENMDAMESSTLELRSRYW					
A/PR/8/34	H	K	V		ET	

Sequences are from Winter and Fields, 1981, and Huddleston and Brownlee, 1982. The two peptide sequences recognized by class I restricted CTL are underlined.

proliferative responses to peptides in the presence of feeder cells and T cell growth factors. The ^{51}Cr release experiments described below were performed either by incubating CTL and target cells in medium containing dissolved peptide (method 1), or by preincubating the target cells in a high concentration of peptide and then washing them extensively before exposure to CTL (method 2; see Experimental Procedures for details).

Recognition of Nucleoprotein Peptides by Murine CTL
Two clones of influenza A specific CTL were investigated for their ability to recognize peptides. Both were derived from C57BL mice (H-2b) and recognized their target antigens in association with the class I MHC molecule D^b.

Cytotoxic T Cell Clone F5
This cloned cell line has been described previously (Townsend et al., 1984a). F5 expressed the Lyt2 positive, L3T4 negative, Thy1 positive phenotype by indirect immunofluorescence with antibodies 53-6.7, GK 1.5, and 97-13 respectively (data not shown). It has been shown to be specific for the 1968 nucleoprotein, and the epitope recognized has been localized to a region of the molecule between amino acids 328 and 388 (Townsend et al., 1985).

Table 1 shows the relevant sequence of the molecule. There are five amino acid differences in the sequences of the nucleoproteins from viruses isolated in 1934 and 1968. As clone F5 could distinguish between these two proteins, one or more of the amino acids that differ were thought likely to form part of the epitope recognized. The Asn-to-His change at position 334 was shown not to play a role because a further deletion mutant of NP that lacked amino acids 255–339 was recognized efficiently by clone F5 (data not shown). Peptides covering the remaining sequence were then tested for their ability to sensitize target cells in the ^{51}Cr release assay, and to stimulate proliferation of the CTL clone in the presence of IL-2.

Figure 1 shows the results in the ^{51}Cr release assay. The three panels at the upper left demonstrate the specificity of clone F5 for the 1968 influenza nucleoprotein; the remaining panels show the level of lysis of the L/D^b target cell in the presence of the indicated peptides. The results showed that recognition by CTL clone F5 could be narrowed down to a 16 amino acid segment represented by peptide 365–380 derived from the 1968 sequence.

The upper half of Figure 2 shows that recognition of peptide 365–380 (1968) by clone F5 was class I MHC restricted. The lower half of Figure 2 demonstrates the effect of titrating out the concentration of peptide 365–380 (1968) while maintaining a constant ratio of CTL clone to target

Figure 1. Recognition by CTL Clone F5 (D^b Restricted) of L/D^b Target Cells in the Presence of Peptides Corresponding to Regions of the NP Amino Acid Sequence

At the top left are the three controls: L/D^b (L cells transfected with the class I MHC gene D^b) in the absence of peptide, NP1 (L cells cotransfected with D^b and the 1968 gene for NP), and L/D^b infected with X31 virus, which expresses the 1934 gene for NP. The remaining panels show the recognition of L/D^b cells in the presence of the indicated peptides at a final concentration of 35 µg/ml (18.8–21.5 µM). The ^{51}Cr release assay was performed by testing CTL at a variety of killer:target (K:T) ratios as described in Experimental Procedures, method 1.

cells of 2:1. The optimum level of lysis was achieved with a concentration of this preparation of peptide in the assay of between 5 × 10^{-8} and 10 × 10^{-6} moles per liter.

Clone F5 also was induced to proliferate, in the presence of IL-2 containing medium and uninfected feeder cells, by peptide 365–380 (1968). Table 2 shows that this peptide induced maximal proliferation whereas peptides 345–360 and 369–382 (1968) had no effect. The result is noteworthy because the optimum concentration of peptide 365–380 (1968) required to induce proliferation by clone F5 was the same as that which induced the plateau level of cytotoxic activity in the ^{51}Cr release assay (Figure 2).

In control experiments clone F5 was tested either by ^{51}Cr release or by thymidine incorporation with a variety

Figure 2. Recognition of Peptide is MHC Restricted and Concentration Dependent

(Top) Recognition by CTL clone F5 of peptide 365–380 (1968) is class I MHC restricted. Recognition of L cells transfected with the class I gene D^b is shown at the left, recognition of untransfected L cells at the right. Target cells were (a) infected with E61-13-H17 virus, which expresses the 1968 NP gene; (b) uninfected; (c) resuspended in peptide 365–380 (1968) at 20 μg/ml (11.5 μM); and (d) resuspended in peptide 369–382 (1968) at 20 μg/ml (12.3 μM).

(Bottom) Recognition by CTL clone F5 of L/D^b cells in the presence of decreasing concentrations of peptide 365–380 (1968). The ⁵¹Cr release assays were done using method 1 and a killer:target ratio of 2:1.

of other peptides derived from the NP sequence, but without any effect (these negative results are summarized in Table 3).

The Optimum Length of Peptide Recognized by Clone F5

In Figures 1 and 2 we have shown that clone F5 recognized the 16 amino acid peptide representing the sequence between residues 365 and 380 of the 1968 nucleoprotein. In later experiments we investigated the effect of reducing the length of this peptide.

A series was made of new peptides of decreasing lengths (during one synthesis), covering the sequences 365–379, 366–379, 367–379, 368–379, and 369–379. These peptides were then compared over a wide range of concentrations in the ⁵¹Cr release assay for their ability to induce lysis of L/D^b and EL4 (H-2b) target cells by clone F5. The experiments were performed using method 1 with the peptides present throughout the assay. The results with EL4 target cells are shown in Figure 3. On the y-axis is shown the level of lysis induced and on the x-axis the molar concentrations of the various peptides used. The

Table 2. Proliferation of Clone F5 with Peptide 365–380 (1968)

Peptide/Virus	Final Concentration (μg/ml)	cpm	SEM
Experiment 1			
E61-13-H17 infected		55,479	1,974
X31 infected		8,658	424
No peptide, uninfected		4,155	38
365–380 (1968)	10 (5.8 μM)	69,093	2,102
365–380 (1968)	1 (0.58 pM)	8,464	491
345–350 (1968)	10 (5.7 μM)	6,301	285
345–350 (1968)	1 (0.57 μM)	5,552	429
Experiment 2			
E61-13-H17 infected		57,391	4,918
No peptide, uninfected		1,045	58
365–380 (1968)	80 (46 μM)	47,431	1,712
365–380 (1968)	40 (23 μM)	69,671	961
365–380 (1968)	20 (11.5 μM)	89,012	2,491
365–380 (1968)	10 (5.8 μM)	83,888	544
365–380 (1968)	5 (2.9 μM)	64,563	497
369–382 (1968)	80 (49.3 μM)	1,210	92
369–382 (1968)	40 (24.6 μM)	1,185	123
369–382 (1968)	20 (12.3 μM)	1,240	213
369–382 (1968)	10 (6.2 μM)	1,212	62
369–382 (1968)	5 (3.1 μM)	1,085	123

The thymidine incorporation assay was performed as described in Experimental Procedures.

L/D^b target cells gave very similar dose-response curves (data not shown).

The most efficient peptide was 366–379 (labeled A in Figure 3). The concentration of this peptide required to induce 20% lysis of the target cells was 6.25×10^{-9} moles per liter. The negative control was 365–380 (1934 sequence), which had no detectable effect up to 2.81×10^{-4} moles per liter (G in Figure 3). An approximately 10-fold greater concentration of the longest peptide of the set, 365–379, was required to induce the same level of lysis as 366–379 (B in Figure 3). The remaining three peptides of the set, 367–379 (C), 368–379 (D), and 369–379 (E), demonstrated a requirement for an approximately 10-fold increase in peptide concentration for each reduction in length by one amino acid to achieve 20% target cell lysis. According to this criterion peptide 369–379 was at least 10⁴-fold less efficient than 366–379 at inducing lysis of EL4 target cells by clone F5 (compare curves A and E in Figure 3).

The set of peptides covering the sequence 365–379 was made during one synthesis on an Applied Biosystems peptide synthesizer. Consequently, each of the products was similar in purity, as assessed by HPLC (approximately 90%; data not shown). The relationships between the dose-response curves for each peptide shown in Figure 3 therefore can be regarded as accurate. The relatively high concentration of peptide 365–380 (1968) required for lysis (Figure 2) and proliferation (Table 2) is related to the fact that this preparation was made separately, using a manual technique, and was correspondingly less pure.

Finally, although 369–379 had a specific effect at concentrations up to 5.12×10^{-4} moles per liter (E in Figure

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Table 9. Summary of CTL Recognition of Nucleoprotein Peptides

			CTL Recognition		
Sequence	Date	Mol. Wt.	Mouse Clone F5	Mouse Clone A3.1	Human Polyclonal MG
325-339[a]	1934	1706	-	NT	-
335-349	1968	1679	-	-	++++
335-349[a]	1934	1651	-	NT	++++
345-360	1968	1766	-	-	-
346-360[a]	1934	1633	-	-	-
357-370[a]	Shared	1516	-	-	+
365-379	1968	1608	++++	NT	NT
366-379	1968	1495	+++++	NT	NT
357-378	1968	1424	+++	NT	NT
366-379	1968	1337	++	NT	NT
369-378	1968	1223	+	NT	NT
365-380[a]	1968	1735	+++	-	-
365-380	1934	1780	-	+++++	-
369-382	1968	1621	-	-	NT
369-382	1934	1555	-	+/-	NT
385-401[a]	1934	1880	-	NT	-
388-411[a]	1934	1458	-	NT	+
410-425[a]	1934	1877	-	NT	+
413-427[a]	1934	1734	-	NT	-
440-455[a]	1934	1845	-	NT	-
448-460[a]	1934	1737	-	NT	-
456-470[a]	1934	1695	-	NT	-
473-487[a]	1934	1628	-	NT	-

[a] These peptides were synthesized manually.
Peptides not synthesized manually were made on an Applied Biosystems peptide synthesizer (see Experimental Procedures). Responses are graded positive (+) or negative (-). NT: not tested.

3), the overlapping peptide 369-382 had only a barely detectable effect (F in Figure 3). The latter peptide was made in a different synthesis but was similar in purity to 359-379.

CTL Clones A3.1, B4, and B8

These three CTL clones were derived from C57BL (H-2b) mice primed and restimulated with the recombinant A virus X31, which contains the NP gene from a 1934 virus isolate (Baez et al., 1980). A3.1 has been described previously (Townsend and Skehel, 1982; Townsend et al., 1983). It also expressed the Lyt2, Thy1 phenotype and had the exact reciprocal specificity to clone F5 described above. A3.1 recognized all A viruses isolated between 1934 and 1943 but not viruses isolated between 1946 and 1979, and experiments with recombinant A viruses showed it to be specific for the 1934 influenza NP (Townsend et al., 1984b). Clones B4 and B8, derived in a separate cloning experiment from A3.1, have been shown to have the same specificity (Wraith, unpublished results).

The reciprocal relationship of the specificities of clones F5 and A3.1 for natural virus isolates led us to test the 365-380 peptide derived from the 1934 NP sequence (see Table 1) for recognition by clones A3.1, B4, and B8. The results for A3.1 in the ^{51}Cr release assay are shown in Figure 4. A3.1 recognized 365-380 (1934) but not 365-380 (1968). In addition A3.1 proliferated in response to 365-380 (1934) but not to 365-380 (1968) (data not shown). Clones B4 and B8 gave the same pattern of activ-

ity as clone A3.1. All of these clones were shown to be restricted through D^b for recognition of peptide 365-380 (1934) (data not shown).

The Effect of Preincubating the Target Cell or the Effector Cell with Peptide Antigens

All of the results described above were from experiments using method 1, where the peptide is present in solution in contact with both CTL and target cells throughout the 6 hr period of the ^{51}Cr release assay.

In Figure 5 the effect of preincubating the target cell is compared with the effect of preincubating clone F5 with peptide 365-380 (1968). Target cells treated in this way were recognized as efficiently as when peptide was present throughout the assay. Figure 5A also shows that CTL recognition of target cells prepulsed with peptide was MHC restricted, as untransfected L cells, which do not express D^b, were not recognized after prior exposure to the peptide.

Figure 5B demonstrates that pulsing the CTL clone with peptide 365-380 had no effect. Clone F5 was not able to recognize untreated L/D^b target cells after prior contact with peptide. Neither did pretreating the CTL clone with peptide have any effect on the recognition of infected, NP transfected (data not shown), or peptide pulsed target cells.

The CTL clone, which expresses the D^b molecule, was examined for the ability to lyse itself when the 365-380 (1968) peptide was present throughout the ^{51}Cr release

Figure 3. Recognition of EL4 (H-2b) Target Cells in the Presence of Decreasing Concentrations of Peptides

A: 366-379 (1968). B: 365-379 (1968). C: 357-379 (1968). D: 366-379 (1968). E: 369-379 (1968). F: 359-382 (1968). G: 365-380 (1934). The ⁵¹Cr release was assayed using method 1 as described in Experimental Procedures. The final concentrations of peptides present during the assay are shown.

Figure 4. Recognition by CTL Clone 43.1 of Peptide 365-380 (1934)

The experiment was set up as described for Figure 1 with the peptides at a final concentration of 95 μg/ml (19.8-21.8 μM). The controls were NP1 (L cells cotransfected with Dᵇ and 1968 NP) and L/Dᵇ infected with X31 (which expresses the 1934 NP). The CTL clone was used at a killer:target ratio of 1.5:1.

Figure 5. Effects of Preincubating Target Cells or CTL Clone with Peptide

(A) Recognition by clone F5 of L/Dᵇ target cells that have been exposed to 100 μg/ml (57.8 μM) of peptide 365-380 (1968) and then extensively washed. (B) Recognition by clone F5 after it has been exposed to 100 μg/ml (57.8 μM) of peptide 365-380 and then washed. Target cells: (○) untreated L/Dᵇ cells; (●) L/Dᵇ cells exposed to peptide 365-380 (1968) then washed four times; (□) untreated L cells; (■) L cells exposed to peptide 365-380 (1968) then washed four times.

assay. F5 was labeled with ⁵¹Cr and incubated for 6 hr in medium containing peptide 365-380 (1968) at between 5 μg/ml and 100 μg/ml (2.9-57.8 μM). No self-lysis occurred (data not shown). Taken with the fact that clone F5 proliferates more efficiently when stimulated with the optimum concentration of peptide than when stimulated with virus infected feeder cells (Table 2), it is apparent that the CTL clone must have a means of avoiding autolysis.

Identification of Nucleoprotein Peptides Recognized by Human CTL

A proportion of humans primed by natural influenza infection can generate CTL in vitro that recognize the viral NP (McMichael et al., 1986). Polyclonal influenza A virus specific CTL prepared from blood donor MG were found to include a major component that was specific for NP. These were then tested on the peptides from NP presently available (summarized in Table 3).

Figure 6 shows the effect of incubating MG CTL with syngeneic target cells in the presence of each peptide during the 6 hr ⁵¹Cr release assay. The ⁵¹Cr-labeled target cells were lysed in the presence of peptide 335-349 (1934) as efficiently as after influenza A virus infection. A

lower level of target cell lysis was induced by peptides 357-370 and 410-425 (1934). It is important to note that MG CTL did not recognize peptides 365-380 from 1934 or 1968.

We have since found that MG CTL which recognize 335-349 (1934) cross-react on the homologous peptide from the 1968 sequence that differs by a Lys-to-Arg change at position 348 (Table 1). This specificity represents the characteristic cross-reactivity displayed by the majority of influenza specific CTL in man (data not shown).

Subsequent experiments showed that optimum conditions for detecting recognition of peptides by MG CTL in the ⁵¹Cr release assay could be obtained by preincubating labeled target cells with peptide (method 2 in Experimental Procedures). The remaining experiments were done in this way.

Human CTL Specific for Peptide 335-349 Are Class I MHC Restricted

To identify the HLA molecules that restricted the recognition of peptide 335-349 (1934) by MG CTL a series of target cells from different blood donors was prepared. The results are shown in Figure 7. MG CTL recognized in-

Figure 6. Screening of Nucleoprotein Peptides in the 51Cr Release Assay with Influenza A Virus Specific Polyclonal Human CTL from Donor MG

At the top the peptides listed in Table 3 are schematically represented. Beneath them is shown the level of target cell lysis that occurred with each peptide at a final concentration of 40 µg/ml (20–37 µM) in the assay. The control target cells were (A) infected with X31 virus and (O) uninfected in the absence of peptide. The 51Cr release assay was performed using method 1 and a killer:target ratio of 50:1 (see Experimental Procedures). The target cells were syngeneic PHA activated lymphoblasts.

Influenza A virus infected cells that shared HLA A1, B37, or B13. Infected target cells that shared HLA A30 or class II molecules (homozygous DR7) were not recognized. Target cells treated with peptide 335–349 (1834) were recognized only when they shared the class I allele HLA B37 with MG. This showed that peptide 335–349 (1834) was recognized by MG CTL only in association with one of his four available allelic class I gene products, whereas live virus infection resulted in the expression of additional determinants on the target cell that were recognized in association with A1 and to a lesser extent with B13. The same results were obtained when either Epstein Barr virus transformed lymphoblastoid cells or PHA stimulated blast cells were used as targets in the 51Cr release assay (data not shown).

As a final specificity control 51Cr-labeled MG cells pretreated with peptide 335–349 (1834) were tested as targets for influenza A specific CTL from an unrelated donor (JJ) who shared only the HLA C6 class I allele with MG. CTL from donor JJ also were able to recognize NP expressed by recombinant vaccinia (data not shown). MG target cells pretreated with peptide 335–349 (1834) were not recognized by JJ CTL. In addition, JJ CTL did not recognize peptide 335–349 (1834) on syngeneic target cells (data not shown).

Human CTL Specific for Peptide 335–349 (1834) Are Inhibited by Monoclonal Antibodies to CD8

Lysis of influenza A virus infected or peptide treated target cells by MG CTL was measured in the presence of saturating concentrations of a variety of monoclonal antibodies known to interfere with T cell recognition or lysis. Figure 8 shows that lysis of peptide treated target cells was inhibited by antibodies to HLA B and C antigens, CD8, CD3, and the alpha chain of LFA-1. No inhibition was obtained

Figure 7. Recognition of Peptide 335–349 by the Human CTL is Class I HLA Restricted

Two experiments are shown (upper and lower parts of the figure). MG polyclonal CTL were tested on PHA activated lymphoblast target cells prepared from a series of related and unrelated individuals. Results are shown as percentage of specific lysis (y-axis) at two killer:target cell ratios. Target cells (■) uninfected; (●) infected with influenza X31 virus; and (O) uninfected and preincubated for 1 hr with peptide 335–349 at 100 µg/ml (method 2 in Experimental Procedures). The HLA A, B, and DR types of MG are shown at the top. The HLA antigens that each target cell shared with MG are shown in parentheses in each panel.

The full HLA types of each target cell were as follows. MG: A1, A30, B13, B37, C6, DR7. FG: A25, A29, B44, C5, DR1, DR7. LG: A1, A29, B44, B37, C6, DR not tested. EG: A26, A30, B44, B13, C5, DR not tested. NR: A1, A3, B8, B60, C3, C7, DR2, DR3. SE: A3, A30, B14, B18, C5, C8, DR2, DR7. PC: A3, A24, B62, B37, C3, C6, DR6. JJ: A2, A11, B35, B50, C4, C6, DR1, DR7.

with antibodies to CD4 or shared determinants of HLA class II molecules. The results with virus infected target cells were similar. These results are typical of class I MHC restricted lysis mediated by CD8 (T8) positive T cells.

Discussion

Cytotoxic T Lymphocytes Recognize Short Synthetic Peptides

We have described influenza A specific CTL from both man and mouse that can recognize short synthetic pep-

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Figure 8. Human CTL That Recognize Peptide 335–349 Are Inhibited by Antibodies to CD8

MG influenza specific polyclonal CTL were tested on autologous MG B lymphoblastoid cells that had been infected with influenza A/X31 virus (left) or uninfected but treated with peptide 335–349 at 100 μg/ml for 1 hr (right). Lysis was tested at a killer:target ratio of 25:1 In the absence of added antibody or in the presence of saturating concentrations of the antibodies shown. Standard errors were all less than 3%. Uninfected MG B lymphoblastoid cells that were not treated with peptide were lysed to 5.2% by the CTL. N.T., not tested.

tides corresponding to regions of the viral NP sequence. Recognition of peptides on the target cell surface was antigen specific and triggered efficient lysis by CTL (Figures 1 and 7). It also induced resting CTL to become responsive to the growth-promoting effect of IL-2 (Table 2).

The epitope recognized by murine CTL clone F5 was studied in detail (Figures 3 and 4). Amino acid changes at positions 372 (Asp to Glu) and 373 (Ala to Thr) abolished recognition of peptide 365–380 by clone F5, but created a new epitope recognized by clone A3.1. These results show that the region of this peptide controlling T cell specificity must contain the residues at positions 372 and 373.

Variation in the length of the peptide containing residues 372 and 373 was shown to have a profound effect on the efficiency with which it could be recognized by clone F5 at the target cell surface. The optimum sequence we have been able to define is the 14 amino acids from position 366 to 379 (A in Figure 3). Variation in length at either end of this peptide was associated with negative shifts in the dose-response curve (B–F in Figure 3).

These experiments with class I restricted CTL were performed using the [61]Cr release assay, which involves a 4–6 hr contact between T cell, target cell, and peptide. The results are clearly related to the wealth of data on class II restricted recognition of peptides obtained in proliferation assays. The optimum concentrations of peptides that induce class I restricted lysis and proliferation are in the same range as a variety of class II restricted responses (reviewed by Hackett et al., 1983). Effects similar to those shown in Figure 3 have been described in several examples of class II restricted recognition of peptides. These include guinea pig T cell recognition of insulin B chain peptide 1–16 (Thomas et al., 1981), a detailed analysis of recognition by murine T cell clones specific for peptide 46–61 of hen egg-white lysozyme (Allen et al., 1985), residues 110–121 of sperm whale myoglobin (Livingstone et al., submitted), and the amino terminus of murine myelin basic protein (Zamvil et al., submitted).

The discussion of these results has centered around the possible conformational and physicochemical restraints on a given immunogenic peptide required for it to interact with both the restriction element (class I or class II MHC molecule) and the T cell receptor (De Lisi and Berzofski, 1985; Heber-Katz et al., 1985). The notion of these interactions has been simplified by the concept of two spatially separate regions of immunogenic peptides, one making contact with the MHC molecule (the "aggretope") and the other with the T cell receptor (the epitope). A variety of experimental data are consistent with these ideas (Werdelin, 1982; Rock and Benacerraf, 1983; Heber-Katz et al., 1982, 1983; Babbitt et al., 1985).

In our example with peptide 365–379 it is tempting to assign the role of epitope to positions 372 and 373. Alterations in these two residues change T cell specificity, but have no apparent effect on MHC restriction (Figures 1, 2, and 4). Residues 365–368 control the efficiency with which the peptide is recognized on the target cell surface, but do not contribute to the specificity of CTL recognition (Figure 3). Residues 365–368 would thus fit the concept of an "aggretope." However, the validity of these assignments in the absence of any structural information remains uncertain.

Recognition of Peptides by CTL Is Class I MHC Restricted

The phenotypes of the CTL we have studied are characteristic of cells that recognize antigens in association with class I MHC gene products (Swain and Dutton, 1980; Dialynas et al., 1983). In each of the three examples, recognition by CTL of peptides on the target cell surface was antigen specific and restricted through a single class I MHC molecule (Figures 2 and 7). This was particularly revealing in the experiments with polyclonal human CTL, in which only one of the three class I molecules recognized on virus infected cells functioned as a restriction element for peptide 335–349 (Figure 7). These observations extend previous work on class I MHC controlled immune response gene effects observed with whole live viruses (Blanden et al., 1979; Vitiello and Sherman, 1983).

The selection of only certain combinations of peptide and class I molecules for recognition by CTL is very similar to the results obtained with nonlytic T cells restricted through class II MHC molecules. Discussion of these effects has focused on the MHC molecules themselves and the T cell repertoire. Polymorphic MHC molecules may bind antigenic peptides with a hierarchy of affinities, and individuals may vary in their T cell repertoires through inherited or acquired diversity in their T cell receptor genes (reviewed by Schwartz, 1985; Shevac, 1982; Zinkernagel and Doherty, 1979; Benacerraf, 1978; Jerne, 1971).

We have shown in Figures 5 and 7 that the peptides recognized by class I restricted CTL can associate with the target cell surface. It is possible, in view of results by Babbitt et al. (1985), that the peptide 365–380 (1960) may bind the D[b] molecule. Both the target cell and the CTL clone express the D[b] molecule (data not shown). Efficient lysis of target cells under conditions where peptide is in contact with both target cell and CTL clone may be explained by

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the resistance of the clone to autolysis. However, as we have no means of detecting peptides on the cell surface other than by CTL recognition, this interpretation is speculative.

The observation that pretreating CTL clone F5 with peptide does not enable it to recognize the untreated L/D target cell implies that peptide 365–380 (1968) does not bind to the T cell receptor in the absence of an appropriate target cell membrane.

A detailed analysis of the binding characteristics of the peptides we have studied will help to clarify these issues.

Do All Somatic Cells Bearing Class I Molecules Present Degraded Viral Proteins to CTL?

We have identified three epitopes of the NP molecule that can be replaced in vitro with synthetic peptides 11 to 16 amino acids in length. Each of the three epitopes exists between amino acids 335 and 385 of the NP sequence. These results, taken with our previous findings (see the Introduction), raise the possibility that degradation or denaturation of NP may take place in the target cell before CTL recognition occurs (Townsend et al., 1985). The alternative explanation is that this region of the molecule is accessible as a segmental or linear epitope in the folded molecule. The three-dimensional structure of NP is not available to judge. Experiments are in progress to compare native with denatured and degraded NP for their ability to sensitize target cells for lysis by CTL. In addition, we are raising antibodies to the peptides recognized by CTL. These may differentiate between native and degraded NP on the surface of transfected or virus infected target cells.

If degradation of viral proteins is required in a target cell prior to CTL recognition, questions arise about where and how this might occur. Recent work has shown that influenza specific CTL restricted through class II molecules exist in both mouse and man (Lukacher et al., 1985; Kaplan et al., 1984). Circumstantial evidence in these experiments was consistent with a processing step by the target cell bearing class II molecules. For instance, presentation of purified NP by lymphoblastoid cell lines to human class II restricted CTL was inhibited by chloroquine (Fleischer et al., 1985).

We have not been able to detect any effect of chloroquine on the recognition by class I restricted murine CTL (which are known to recognize peptide 365–380) of virus infected or NP transfected target cells (Gotch, unpublished work). Initial results with class I restricted human CTL show that NP purified from influenza virus particles is not capable of sensitizing lymphoblastoid target cells for lysis by MG CTL (data not shown).

If a proteolytic system does exist that is involved in presentation of antigen to class I restricted CTL, it should be present in all the cell types capable of being recognized by CTL, act on newly synthesized viral proteins, and be resistant to the effects of chloroquine and other agents thought to inhibit lysosomal degradation of proteins.

In conclusion, we have demonstrated that the epitopes of influenza NP recognized by class I restricted CTL on the surface of their target cells can be defined with short synthetic peptides. Preliminary evidence suggests that the peptides recognized by CTL can associate with the target cell membrane. The results are consistent with the suggestion that degradation of NP may be required in the target cell prior to CTL recognition. All of the phenomena we have described with class I restricted CTL are closely related to well established findings with class II restricted T cells.

Experimental Procedures

Murine CTL Clones

Clones F5 and A3.1 were isolated from C57BL/6 (obtained from OLAC) or C57BL (obtained from NIMR Mill Hill) by limiting dilution as described previously (Townsend et al., 1983, 1985). They were maintained in vitro by stimulation with A virus infected syngeneic spleen cells in TCGF conditioned medium exactly as described previously (Townsend and Skehel, 1984). For some experiments clone F5 was maintained with uninfected feeder cells and TCGF conditioned medium containing peptide 365–390 (1968) at a final concentration of 10 pg/ml. The optimum growth-promoting activity of peptide was assessed by prior titration in a thymidine incorporation assay.

Induction and Testing of Human Polyclonal CTL

The method used was based on that described previously (McMichael and Askonas, 1978) with some modifications (McMichael et al., 1986). Peripheral blood lymphocytes (PBL) were incubated in RPMI 1640 medium (Gibco) in the absence of serum with influenza X31 virus for 1 hr at 37°C. Fetal calf serum was added to 5% and the flask incubated for a further 7 days. The cultures were then harvested and resuspended in RPMI 1640, 10% FCS for use in the ⁵¹Cr release assay. Target cells were prepared and chromium labeled as previously described (McMichael and Askonas, 1978; McMichael et al., 1986). They were either autologous Epstein Barr virus transformed B lymphoblastoid cell lines or PBL activated for 3 days with phytohaemagglutinin at 2 µg/ml. For the peptide experiments, methods 1 and 2 described for the mouse CTL experiments were used.

⁵¹Chromium Release Assay

A standard procedure was used (Zweerink et al., 1977; Townsend et al., 1983, 1984; McMichael and Askonas, 1978) with the following modifications for testing peptides.

Method 1
Peptides were made up in RPMI medium containing 10% FCS and 10 mM Hepes buffer at pH 7.4 (RPMI/10) at 4 mg/ml. This stock solution was then diluted in RPMI/10 to four times the final concentration required in the assay and dispensed in 0.05 ml aliquots to experimental and control wells of 96 well microtiter trays. Target cells were harvested, resuspended in 0.5 ml RPMI/10 containing 100 µCi of ⁵¹Cr, incubated at 37°C for 90 min, then washed three times in 10 ml PBS, and once in RPMI/10, and resuspended in RPMI/10. Labeled target cells (1 × 10⁴ or 2 × 10⁴) in 0.05 ml RPMI/10 were added to round or flat-bottomed wells of 96 well microtiter trays containing 0.05 ml aliquots of peptides. Additions to this mixture were as follows: to experimental wells, CTL in 0.1 ml to make up the killer:target (K/T) ratios shown in the figures; to control wells, 0.1 ml of RPMI/10; and to total release wells, 0.1 ml of 5% Triton-X 100. The assay was then performed as previously described.

Method 2
Target cells were harvested, washed twice in serum free RPMI, then resuspended in 0.4 ml serum free RPMI containing 100 µCi ⁵¹Cr and peptide at a final concentration of 100 µg/ml (approximately 60 µM). The mixture was then incubated for 90 min at 37°C, washed three times in 10 ml PBS, washed once in 5 ml RPMI/10, and resuspended in RPMI/10. Labeled target cells (1 × 10⁴ or 2 × 10⁴) were then dispensed in 0.1 ml aliquots in microtiter trays. The assay was then continued as previously described. A minor modification was used in some of the experiments with human CTL. Target cells were labeled with ⁵¹Cr for 90 min in the absence of peptide, washed twice in serum free RPMI, then resuspended in 0.4 ml serum free RPMI containing peptide at 100 µg/ml for 1 hr at 37°C. The cells were then washed three times and dispensed in microtiter trays. The assay was then con-

tinued as previously described. These two variations of method 2 gave identical results.

Percentage of specific ^{51}Cr release was calculated as follows:

$$\frac{(\text{Release by CTL} - \text{medium release})}{(2.5\% \text{ Triton release} - \text{medium release})} \times 100$$

Each experimental point was measured in duplicate against quadruplicate controls to medium alone. Spontaneous ^{51}Cr release by targets in medium alone varied between 10% and 17% for murine target cells and between 15% and 28% for human target cells of counts released by Triton.

Dose-Response Titrations of Peptides

For the dose-response curves in Figure 3, EL4 (H-2b) target cells were used. Peptides were dispensed in round-bottomed 96 well sterile microtiter plates as described above in 0.05 ml, and a series of nine doubling dilutions made over a dose range optimized by trial and error in previous experiments. ^{51}Cr-labeled EL4 cells (10^4) in 0.05 ml were added to each well followed by 1.5×10^4 CTL clone F5 in 0.1 ml. The assay was then performed as previously described. The results in Figure 3 were calculated in the following way to normalize the titrations to the 0% line:

$$\frac{(\text{Release in presence of CTL and peptide} - \text{release by CTL alone})}{(2.5\% \text{ Triton release} - \text{release by CTL alone})} \times 100$$

The spontaneous release of ^{51}Cr from target cells in the presence of CTL but no peptide was 14% of the counts released by Triton. Within the dose ranges shown in Figure 3 none of the peptides alone had any detectable effect on the spontaneous release of ^{51}Cr from target cells.

The Thymidine Incorporation Assay

CTL clone F5 was fed with virus infected syngeneic spleen cells and TCGF conditioned medium as previously described (Townsend et al., 1983; Townsend and Skehel, 1984). Three to four days later the responding cells were split 1:2 and fed with fresh TCGF conditioned medium. This procedure was repeated every 3 to 4 days for at least 14 days. By this time the CTL clone stopped dividing, even when fed with fresh conditioned medium. Further cell division was absolutely antigen dependent.

Peptide dilutions were prepared from a stock solution of 4 mg/ml in RPMI/10 that had been filter sterilized. Diluted peptides or medium alone were then dispensed in 0.05 ml triplicate aliquots to wells of round-bottomed 96 well sterile microtiter plates. To these aliquots was added 0.15 ml RPMI/10 containing 10^4 clone F5 cells; 5×10^5 uninfected or E61-13-H17 infected, 2000 rad irradiated syngeneic spleen cells; and 0.05 ml of a Con A stimulated rat spleen cell supernatant (Townsend and Skehel, 1984). The cultures were incubated for 3 days at 37°C in 5% CO_2 and pulsed with 1 µCi of ^{3}H thymidine for 6 hr; the cells were then harvested on a Titertek 550 cell harvester. The ^{3}H thymidine incorporation by the CTL clone was measured by scintillation counting. Triplicate wells were set up for each peptide dilution, and the mean and standard error of the mean were calculated for each point.

Peptide Synthesis

Some peptides in Table 3 (see footnote a) were synthesized manually by Merrifield's solid-phase method (Merrifield, 1963) using t-Boc suitably protected amino acids and the appropriately derivatized amino acid resin (Sigma). N,N'-dicyclohexylcarbodiimide (DCC) was used as the coupling agent. The peptide was cleaved off the resin with hydrogen bromide and trifluoroacetic acid, then desalted on G15 Sephadex with 50% acetic acid. When arginine and cystine were present the peptides were further deprotected in sodium and liquid ammonia, then desalted. The crude freeze dried material was analyzed on a Beckman 121B amino acid analyzer, and by high pressure liquid chromatography (HPLC).

The remaining peptides in Table 3 were synthesized by solid phase techniques on an Applied Biosystems peptide synthesizer, model 430A. Commercially available amino acid phenylacetamidomethyl resins and t-Boc suitably protected amino acids were used. All

couplings were performed using a 2.5 molar excess of t-Boc amino acid and DCC over the number of milliequivalents of amino acid on the resin. In the case of Asn and Gln a 2.5 molar excess of the amino acid, DCC, and N-hydroxytriazole was used. The peptides were deprotected and removed from the resin simultaneously by treatment with anhydrous hydrogen fluoride in the presence of anisole, dimethyl sulfide, and indole. The peptides were separated from the various organic side products by extraction with ether and isolated from the resin by extraction with 5% acetic acid and subsequent lyophilization. The purity of the crude product was determined by HPLC on a C-18 reverse phase column. All the peptides synthesized by this method contained > 90% of the desired product.

Influenza Virus Strains

The recombinant A viruses X31 (Baez et al., 1980) and E61-13-H17 (Lubeck et al., 1979, where this virus is referred to as recombinant S3; P. Palese, personal communication) differ only in the origin of their genes for nucleoprotein. E61-13-H17 contains the 1968 NP gene from A/HK/8/68, and X31 contains the 1934 NP gene from A/PR/8/34. Virus was grown in the allantoic sacks of 11 day old embryonated chicken eggs, and stored as infectious allantoic fluid at −70°C.

Human Blood Donors

All donors were healthy volunteers. EG and LG were daughters of MG and FG. JJ, NR, PC, and SE were unrelated. HLA types were determined by the standard technique of the National Institutes of Health.

Inhibition of Human CTL with Monoclonal Antibodies

The monoclonal antibodies used were as follows: UCHT1, anti-CD3, a gift from Dr. P. C. L. Beverley, University College, London; anti-Leu 3a, anti-CD4, a gift from Dr. R. Evans, Memorial Sloan-Kettering Institute, New York; B9.4, anti-CD8, a gift from Dr. C. Mawas, Centre d'immunologie, Marseille; SGТ71, anti-HLA class II, a gift from Dr. S. Goyert, Hospital for Joint Diseases, New York; PA2.6 anti-HLA class I, a gift from Dr. P. Parham, Stanford University; MHMS anti-HLA B and C; and MHM24 anti-LFA-1α chain. Antibodies were added to the mixture of killer and target cells at the initiation of the chromium release assay to a concentration of 1/200 ascites. This was determined to be in excess of the concentration required to saturate all the antigenic sites of the cells in the assay.

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Localization of Epstein-Barr Virus Cytotoxic T Cell Epitopes Using Recombinant Vaccinia: Implications for Vaccine Development

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Summary

There is considerable interest in designing an effective vaccine to the ubiquitous Epstein-Barr virus (EBV). An important role for EBV-specific cytotoxic T lymphocytes (CTLs) in eliminating virus-infected cells is well established. Limited studies using a small number of immune donors have defined target epitopes within the latent antigens of EBV. The present study provides an extensive analysis of the distribution of class I–restricted CTL epitopes within EBV-encoded proteins. Using recombinant vaccinia encoding individual EBV latent antigens (Epstein-Barr nuclear antigen [EBNA] 1, 2, 3A, 3B, 3C, LP, and LMP 1), we have successfully localized target epitopes recognized by CTL clones from a panel of 14 EBV-immune donors. Of the 20 CTL epitopes localized, five were defined at the peptide level. Although CTL clones specific for nine epitopes recognized both type 1 and type 2 transformants, a significant number of epitopes (7/16 epitopes for which EBV type specificity was determined) were detected only on type 1 EBV transformants. Vaccinia recombinants encoding EBNA 3A and EBNA 3C were recognized more frequently than any other vaccinia recombinants used in this study, while no CTL epitopes were localized in EBNA 1. Surprisingly, epitope specificity for a large number of EBV-specific CTL clones could not be localized, although vaccinia recombinants used in this study encoded most of the latent antigens of EBV. These results suggest that any EBV vaccine based on CTL epitopes designed to provide widespread protection will need to include not only latent antigen sequences but also other regions of the genome. The apparent inability of human CTLs to recognize EBNA 1 as a target antigen, often the only latent antigen expressed in Burkitt's lymphoma and nasopharyngeal carcinoma, suggests that EBV-specific CTL control of these tumors will not be feasible unless the down-regulation of latent antigens can be reversed.

Protective immunity to viral infection requires the development of memory T cells that recognize viral antigens in association with class I MHC. Earlier studies on influenza virus first highlighted the important role of CD8⁺ CTLs, which recognize virus-encoded proteins in the form of short peptides (1). Since it is well established that immunization with whole viral proteins is unable to elicit an efficient CTL response, interest has been directed towards peptide vaccines based on defined epitope sequences. This is particularly the case with oncogenic viruses, since individual viral genes introduced in recombinant vectors have the potential to initiate tumorigenic processes. Thus, it is important to determine the distribution of these epitopes within viral proteins and the frequency with which infected cells from a significant cohort of immune donors present these epitopes in association with MHC class I alleles. Because of the potential im-

portance of CTL epitopes in the future development of a vaccine to EBV, a herpes virus with known oncogenic potential, there is considerable interest in defining these EBV-encoded molecules recognized by CTLs.

EBV is the etiological agent of infectious mononucleosis (IM)¹ and is associated with Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) (2), lymphomas in immunocompromised individuals (3), and more recent evidence suggests an association with Hodgkin's lymphoma (4). Th

types of EBV (1 and 2, also referred to as A and B) are recognized that show DNA sequence divergence within the BamHI WYH and E regions of the genome (5–7). In vitro, the virus transforms human B cells into lymphoblastoid cell lines (LCLs), which express a limited number of viral gene products, including a family of Epstein-Barr nuclear antigens (EBNA): 1, 2, 3A, 3B, and 3C, leader protein (EBNA LP), latent membrane proteins (LMP 1 and 2), and terminal proteins (TP 1 and 2) (5). An alternative nomenclature also in current use designates the EBNA family as EBNA 1, 2, 3, 4, and 6, and EBNA-5/LP (4, 5). In contrast, latent antigen expression in BL and NPC is restricted to EBNA 1 (in some instances LMP is also expressed in NPC) (8).

In all previously infected individuals, the virus persists for life as a latent infection in B cells and is apparently restrained by a population of EBV-specific CTLs (9, 10). This CTL response is a classic virus-specific response (CD8, class I restricted) (10), though CD4 class II–restricted cells have also been described (9). The key observation in defining the first peptide epitope recognized by EBV-specific CTLs was that in certain donors it was possible to exploit the allelic polymorphism in the EBNA proteins between type 1 and type 2 EBV by isolating type 1–specific CTL clones (11). These clones provided an opportunity to screen selected EBNA peptides for reactivity on type 2 transformants. This led to the definition of an EBV-specific CTL epitope that was present on type 1 but not type 2 transformants (12). A second epitope, derived from EBNA 3C and present on both type 1 and type 2 transformants, has also been described (13). While this approach was very successful in defining a limited number of CTL epitopes, their overall distribution within EBV latent antigens was largely undetermined.

Two recent technical advances from our laboratories have facilitated this study. First, the construction of recombinant vaccinia capable of expressing individual EBV latent antigens, and second, the establishment of an EBV-negative host cell (anti-μ B cell blasts) for these recombinant vaccinia (14, 15). In the present report, we have localized EBV CTL epitopes recognized by multiple CTL clones from a panel of immune donors to generate the first comprehensive analysis of the distribution of CTL epitopes within the EBV latent antigens. This approach was combined with peptide epitope mapping, which permitted the identification of a number of new CTL epitopes. Moreover, since EBV infection is associated with BL and NPC, another important objective of this study was to determine whether any CTL epitopes are localized within EBNA 1 and/or LMP.

Materials and Methods

Establishment and Maintenance of EBV-transformed Cell Lines. LCLs were established from a panel of healthy EBV-seropositive donors listed in Table 1 by exogenous virus transformation of peripheral B cells using type 1 (B95.8 and IARC-BL74) or type 2 (Ag876) EBV isolates (11), and were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin plus 10% FCS (growth medium). LCLs were desig-

nated with the donors initial followed by the transforming virus source (e.g., LC/B95.8 designates B lymphocytes from donor LC transformed with virus from the B95.8 cell line).

Generation of Anti-μ B Cell Blasts. Unfractionated mononuclear (UM) cells were separated on Ficoll/Paque (Pharmacia, Uppsala, Sweden) and depleted of T cells using E-rosetting (16). The enriched B lymphocytes were cultured in growth medium containing anti-IgM (μ chain specific) coupled to acrylamide beads (Bio-Rad Laboratories, Richmond, CA), human rIL-4 (50 U/ml; Genzyme, Boston, MA), and highly purified human rIL-2 from *Escherichia coli* (20–40 U/ml) (17, 18). After 48–72 h, B cell blasts were suspended in growth medium supplemented with rIL-2 (20–40 U/ml). The B cells continue to divide two to three times/wk for 3 wk in the presence of rIL-2. These cells are referred to as anti-μ B cell blasts.

Vaccinia Virus Recombinants. Recombinant vaccinia constructs for different EBNA genes have been previously described (14, 15, 19). All EBV sequences were derived from the B95.8 strain of virus. The EBNA 3A sequence was derived from the cDNA Clone T216 (7), which consists of the 5' portion of the EBNA 3A coding sequence, crossing the splice site and extending to the EcoRI site located at position 93166. This plasmid was digested with PstI and collapsed onto itself to remove 5' noncoding sequences. The resulting plasmid, T216P, was opened at the EcoRI site and ligated to the genomic EcoK fragment derived from plasmid PMH36, recreating the intact full-length EBNA 3A coding sequence. The EBNA 3A coding region was excised with PstI and EcoRV and ligated to Psc11 at the SmaI site. The EBNA 3B was derived from the cDNA clone PMLPT7. The portion of the open reading frame crossing the splice junction was excised with SpeI and XbaI and inserted into SpeI-digested pBluescript. The resulting plasmid was digested with SpeI and EcoRI and ligated to a 440-bp genomic fragment from EcoRI (95243) to SpeI (95683). The resulting plasmid (pBS:E3B) was digested with EcoRI and XbaI to release full-length coding region of EBNA 3B and ligated to the SmaI site of PSC11.

All constructs had the potential to encode the relevant full-length EBV protein except for EBNA 2 deletion mutants. The diagrammatic representation of EBNA 2 deletion mutants is shown in Fig. 1. All constructs utilize the authentic start and stop codons. All constructs are under the control of vaccinia virus P7.5 promotor, except EBNA 1, which has been described elsewhere (19). A vaccinia virus construct made from insertion of the pSC11 vector alone and negative for thymidine kinase (Vacc.TK⁻) was used as control.

Source of Generation of EBV-specific CTL Clones. UM cells (10⁶/ml) from each donor were cultivated with irradiated (8.000 rad) autologous type 1 (B95.8) LCLs (responder to stimulator ratio of 200:1) in 2-ml culture wells (Linbro Chemical Co., Hamden, CT) for 3 d in growth medium. In the case of donors LC and IM, CTL clones were also established after stimulation with BL74-transformed autologous LCLs. CTL clones generated by seeding in 0.35% agarose were established from these donors and maintained as described earlier (9, 11). Colonies were harvested after 3 d and amplified in culture with biweekly restimulation with rIL-2 and autologous LCL.

Cytotoxicity Assay on LCLs. CTL clones from each donor were screened in a standard 5-h ⁵¹Cr release assay (at an E/T ratio of 5:1 or 10:1) for specific reactivity against autologous types 1 and 2 and allogeneic type 1 LCLs as previously described (11). Clones were designated as being EBV-specific on the basis of recognition of the autologous type 1 LCL and lack of recognition of MHC-unrelated LCLs and autologous anti-μ B cell blasts and/or PHA blasts.

Cytotoxicity Assay on Recombinant Vaccinia Virus–infected Targets.
Anti-μ B cell blasts or type 2 LCLs were infected with recombinant vaccinia viruses at a multiplicity of infection (M.O.I.) of 10:1 for 1 h at 37°C as described earlier (14). After 14–16 h, cells were washed with RPMI 1640 and incubated with ^{51}Cr for 90 min, washed three times, and used as targets in a standard 5-h ^{51}Cr-release assay as described above. The effector cells were added to the assay at E/T ratios between 5:1 and 10:1. To confirm the expression of EBV antigens in anti-μ B cell blasts and/or LCLs after recombinant vaccinia infection, the infected cells were also processed for immunoblotting and immunofluorescence (14).

Screening of CTL Clones for Peptide Epitope Specificity. To identify the CTL epitopes recognized by EBV-specific CTL clones from each donor, a series of peptides from EBNA or LMP 1 were synthesized (10–15 amino acids) (20) based on the known sequence of the B95.8 strain of EBV. Peptides selected were primarily based on the results of recombinant vaccinia CTL assays and those that corresponded to predicted algorithms (21, 22). Peptides were dissolved in RPMI 1640 and distributed into U-bottomed microdilution plates (200 μg/ml, 20 μl/well) and frozen at −70°C until required. ^{51}Cr-labeled anti-μ B cell blasts were added to each well (2 × 10^5/ml, 50 μl/ml) and incubated at 37°C. After 1 h, 130 μl of cloned autologous CTLs were added to the reaction mixture (final E/T ratio as indicated), and the assay was conducted as described above.

Results

Localization of EBV CTL Epitopes in EBV-immune Donors. A total of 362 clones were isolated from a panel of 14 healthy EBV-immune donors after stimulation with irradiated autologous B95.8- or BL74-transformed LCLs. Of these, 212 were EBV-specific CTLs. The proportion of these specific CTL clones from each donor varied from 100% (17/17 for donor DM) to as low as 16% (8/51 for donor AS) (Table 2).

To define the antigen specificity of the 212 EBV-specific CTL clones, autologous anti-μ B cell blasts or type 2 LCLs were infected with recombinant vaccinia expressing individual EBV latent antigens (and Vacc.TK$^-$) and used as targets in a ^{51}Cr release assay. The reactivity of five EBV-specific clones from one of the 14 donors (DD) is illustrated in Fig. 2, and demonstrates that two of these clones recognize Vacc.EBNA 3A (CTL5 and CTL13), two recognize Vacc.LPM1 (CTL8 and CTL10), while the antigen specificity of one clone (CTL9) was not defined by the panel of vaccinia constructs. Table

Table 1. HLA Antigen (Class I) Type of the EBV-immune Donors Included in this Study

Donor	HLA typing
LC	A1, B8, B18
IM	A1, A11, B8, B51
DM	A24, A29, B44, B47
CM	A11, A24, B7, B44
PM	A11, A29, B7, B44
AS	A2, A24, B51, B62
NB	A2, A24, B7, B35
DD	A1, A3, B8, B40
LX	A24, B15, B38
LL	A2, B7, B44
CS	A2, A23, B35, B44
SJ	A2, A3, B7, B44
JA	A2, A11, B7, B15
JS	A1, A2, B8, B51

2 presents a summary of the EBNA/LMP 1 vaccinia constructs recognized by CTL clones from each of the 14 donors. All vaccinia constructs except Vacc.EBNA 1 and Vacc.EBNA 3B were recognized by EBV-specific CTL clones. However, it should be emphasized that recognition of Vacc.EBNA 3B-infected anti-μ B cell blasts by EBV-specific CTL clones was assessed in only five donors (Table 2). A dominant response through a single vaccinia construct was observed with some donors (DM, CM, and CS recognized primarily Vacc.EBNA 3C) (Table 2). An important feature of these results was that the majority (145/212) of EBV specific clones failed to recognize any of the latent antigens encoded by vaccinia constructs (Table 2). This was particularly evident in the case of donors PM and JA, where none of the clones recognized cells infected with any of the vaccinia constructs.

MHC Class I Restriction of Vaccinia-localized CTL Epitopes. Of the 212 EBV-specific CTL clones investigated in the present study, 20 distinct epitopes were localized using vaccinia constructs. CTL clones specific for seven of these epitopes were type 1 specific, while clones specific for nine

Figure 1. Diagrammatic representation of B95.8 EBNA 2 sequence and of vaccinia constructs encoding EBNA 2 and its deletion mutants. The construct encoding the full-length EBNA 2 is designated (■), while the regions deleted from EBNA 2 are designated (□). Details for the preparation of these mutants has been published earlier (15). Each recombinant vaccinia had the capacity to encode truncated EBNA 2 proteins with the following amino acid residues deleted: Vacc.HB has amino acid residues 19–118 deleted; Vacc.BB has amino acid residues 151–327 deleted; Vacc.St has amino acid residues 203–237 deleted; Vacc.Sp has amino acid residues 251–384 deleted; and Vacc.PP has amino acid residues 405–480 deleted.

Table 2. *Recognition of Vaccinia Recombinants Encoding EBV Latent Antigens by EBV-specific CTL Clones from Immune Donors*

Donor	No. of clones	EBV specific clones[t]	Vacc. EBNA 1	Vacc. EBNA 2[s]	Vacc. EBNA 3A	Vacc. EBNA 3B	Vacc. EBNA 3C	Vacc. EBNA LP	Vacc. LMP 1
LC	47	44	1	2	7	–	–	–	1
IM	26	15	i	–	4	–	–	–	–
DM	17	17	i	–	–	NT[1]	12	–	–
CM	29	19	i	–	1	NT	·6	–	–
PM	25	17	–	–	–	NT	–	–	3
AS	51	8	–	–	1	NT	–	1	1
NB	42	8	–	5	1	NT	–	–	3
DD	21	17	–	–	3	–	–	–	–
LX	14	5	–	–	3	–	–	–	–
LL	19	16·		–	1	NT	2	2	1
CS	15	12	–	–	–	NT	4	–	–
SJ	24	19	–	–	1	NT	–	–	–
JA	12	4	–	–	–	NT	–	–	–
JS	20	11		2	–	–	–	–	–
Total	362	212	0	9	22	0	24	3	9

* Total number of clones tested for cytolytic activity.
t This column summarizes the number of EBV-specific CTL clones isolated from each individual donor.
s This column refers to the number of clones recognizing autologous anti-μ B cell blasts infected with recombinant vaccinia encoding EBNA 2.
l No clones reactive to vaccinia construct.
1 Not tested.

other epitopes recognized both type 1 and type 2 transformants. The type specificity of four epitopes was undefined (Table 3). The HLA restriction of the specific epitopes was determined by comparing the lysis of autologous LCLs and allogeneic LCLs sharing one or more alleles (Table 3). CTL clones restricted through eight different alleles were observed while the restricting alleles for five EBV CTL epitopes were undetermined (Table 3). An important feature of these results was that different clones restricted through HLA A2, B7, B40, B8, and B51 each recognized epitopes included in two different latent antigens (Table 3). This observation implies that a single allele can present two distinct EBV CTL epitopes.

More precise localization of CTL epitopes within EBNA 2 was facilitated by the availability of deletion mutants encoding truncated EBNA 2 proteins. In all, nine EBNA 2-specific CTL clones restricted through three different alleles (HLA A2, B18, and B7) were isolated (Table 3). The A2-restricted clones from donors JS and NB recognized four deletion mutants (Vacc.BB, Vacc.St, Vacc.PP and Vacc.Sp) with a level of lysis comparable with Vacc.EBNA 2. In contrast, the Vacc.HB mutant, which had a deletion affecting the NH$_2$ terminus of EBNA 2 protein (Fig. 1), was not recognized by these clones. Data from one such A2-restricted CTL clone from donor NB is shown in Fig. 3 a. In contrast, the HLA B18-restricted EBNA 2–specific CTL clones (from donor LC), failed to recognize Vacc.Sp and Vacc.BB deletion mutants, which had overlapping deletions for amino acids 251–327 of EBNA 2 protein (Figs. 1 and 3 b).

Based on the results obtained from the vaccinia experiments, EBV peptides from respective EBNA/LMP 1 regions were screened for their ability to sensitize autologous anti-μ B cell blasts for EBV-specific CTL lysis. In some instances, this involved the selection of 15–20-mer peptides from individual

Figure 2. Specific lysis by EBV-specific CTL clones (CTL5, 8, 9, 10, and 13) from donor DD of autologous LCLs (DD/B95.8) and Vacc. EBNA 1, 2, 3A, 3B, 3C, LP, LMP 1, and TK⁻-infected DD anti-μ B cell blasts. Anti-μ B cell blasts were infected for 12–14 h (M.O.I., 10:1) with vaccinia constructs and processed for standard ⁵¹Cr release assay. Vacc.TK⁻ was used as a control recombinant vaccinia. Results are expressed as percent specific lysis observed in a standard 5-h chromium-release assay. An E/T ratio of 5:1 was used throughout the assay.

Table 3. *Summary of the Distribution of MHC Class I–restricted EBV CTL Peptide Epitopes within EBV Latent Antigens Localized by Recombinant Vaccinia*

EBV antigen recognized	HLA restriction	Peptide epitope	EBV type specificity	Donor(s)
EBNA 2	HLA A2	DTPLIPLTIF[*]	Type 1	JS and NB
EBNA 2	HLA B18	PRSPTVFYNIPPMPL[1]	Type 1	LC
EBNA 2	HLA B7	Undefined	Type 1 and 2	NB
EBNA 3A	HLA B8	FLRGRAYGL[5]	Type 1	LC and IM
EBNA 3A	HLA B7	Undefined	Type 1 and 2	NB and SJ
EBNA 3A	HLA A11 or A24	Undefined	Type 1	CM
EBNA 3A	HLA B51	Undefined	Type 1	AS
EBNA 3A	Undefined	Undefined	ND	LX
EBNA 3A	HLA 40	Undefined	ND	DD
EBNA 3A	Undefined	Undefined	Type 1 and 2	LL
EBNA 3C	HLA A24 or B44	RGIKEHVIQNAFRKA[1]	Type 1	CM and DM
EBNA 3C	HLA B44	EENLLDFVRF[1]	Type 1 and 2	DM, CM, CS, and LL
EBNA 3C	Undefined	Undefined	Type 1 and 2	CS
EBNA LP	Undefined	Undefined	Type 1 and 2	NB and LL
LMP 1	HLA A2	Undefined	Type 1 and 2	LL and NB
LMP 1	HLA B51	Undefined	Type 1 and 2	AS
LMP 1	HLA B40	Undefined	ND	DD
LMP 1	HLA A24	Undefined	Type 1 and 2	AS
LMP 1	Undefined	Undefined	Type 1	LC
LMP 1	HLA B8	Undefined	ND	DD

[*] Reference 23.
[1] This study.
[5] Reference 12.
[1] Reference 13.

latent antigens that corresponded to predicted algorithms (20–22). However, to define epitopes from EBNA 2. peptides were selected that corresponded to the relevant vaccinia deletion mutant. Of the 20 distinct CTL epitopes localized in this study, five were defined at the peptide level while the other 15 remained undefined (Table 3). Of these five epitopes, three have been previously published (12, 13, 23), while new epitopes from EBNA 2 and EBNA 3C are presented in Table 3. The results of experiments that define these new epitopes are included in Fig. 4, *a* and *b*. The HLA B18–restricted EBNA 2–specific clone recognized autologous anti-μ B cell blasts sensitized with peptide PRSPTVFYNIPPMPL (residue number 276–290), while the EBNA 3C-specific clone. restricted through either HLA A24 or B44, recognized peptide RGIKEHVIQNAFRKA (residue number 332–346) (Fig. 4, *a* and *b*).

Discussion

There is convincing evidence that EBV-specific memory T cells are responsible for controlling the level of EBV-positive

B lymphocytes, which all healthy seropositive individuals carry for life after primary infection with the virus. Experimental support for the existence of this protective memory T cell population came from the observation that in virus-infected cultures of mononuclear lymphocytes from seropositive (but not seronegative) donors, the initial proliferation of EBNA-positive B cells was followed by a complete T cell–dependent regression of growth such that LCLs could not be established from subcultures (24, 25). This observation suggested that the latent antigens expressed by these LCLs were a source of target antigens. Indeed, we have recently demonstrated the existence of CTL epitopes within three of the EBNA proteins by screening individual clones against a panel of peptides derived from a range of latent antigens. Although these studies identified several CTL epitopes (12, 13, 23), there has been no previous attempt to determine the relative distribution of CTL epitopes within EBV proteins, recognized by healthy immune donors expressing an array of MHC class I alleles. Such an evaluation is a mandatory prerequisite for any future CTL-based vaccine to EBV. The present study provides an extensive analysis of the distribution of CTL epi-

Figure 3. Functional analysis of EBNA 2-specific CTL clones from donors NB (*a*) and LC (*b*) generated from an in vitro stimulation with autologous B95.8 LCLs and tested on autologous type 1 LCL (NB/B95.8 or LC/B95.8) and anti-μ B cell blasts infected with recombinant vaccinia carrying EBNA 2 gene deletions (Vacc.HB, BB, St, PP, and Sp). Vacc.EBNA 2, or Vacc.TK⁻. Results are expressed as in Fig. 2. For details on the amino acids deleted from each recombinant vaccinia, see legend to Fig. 1.

Figure 4. Recognition of peptide-sensitized autologous anti-μ B cell blasts by EBV-specific CTL clones from donors LC (*a*) and CM (*b*). ³¹Cr-labeled anti-μ B cell blasts were presensitized with each peptide for 1 h and subsequently screened in a CTL assay using the relevant CTL clone. An E/T ratio of 5:1 was used. The results are expressed as in Fig. 2.

topes within viral-encoded proteins. Using recombinant vaccinia encoding individual EBV latent antigens, we have successfully localized target epitopes recognized by CTL clones from a panel of EBV-immune donors.

The location of CTL epitopes within the seven latent antigens, for which vaccinia constructs were available, was unevenly distributed. In particular, EBNA 3A and EBNA 3C were common sites for CTL recognition (11/14 donors had CTL reactivity to either of these antigens), while no epitopes were localized in EBNA 1 and EBNA 3B. Since, in the present study, Vacc.EBNA 3B was not available to test the reactivity of CTL clones from nine donors, it is not possible to draw any conclusions about the occurrence of epitopes in this protein. The immunodominance of the EBNA 3 family of proteins as a source of EBV CTL epitopes seen in this study using EBV-specific CTL clones has been confirmed in a similar study using polyclonal EBV-specific T cells (19). In all, 20 distinct CTL specificities restricted through eight different class I alleles have been defined. Interestingly, HLA A2, B7, B8, B40, and B51 alleles were each shown to present two distinct CTL epitopes derived

from different latent antigens. The ability of single MHC alleles to present multiple epitopes has also been reported with HIV (26).

Surprisingly, the specificity of a large number of EBV-specific CTL clones (68%) using the available panel of vaccinia constructs could not be defined. A possible explanation for this result is that the undefined epitopes are located within EBV latent antigens other than those encoded by the vaccinia constructs available for this study; for example, terminal proteins (TP 1 and TP 2), LMP 2, or as yet unidentified latent antigens. Alternatively, antigens associated with the EBV replicative cycle could also include CTL epitopes. These antigens are detected in only a small proportion of latently infected cells by conventional techniques. However, the exquisite sensitivity by which CTLs recognize peptide fragments (27) from viral antigen raises the possibility that low levels of replicative antigens could be processed and presented as target epitopes on LCLs. It is relevant to point out that in another related study, EBNA 3B and LMP 2 were identified as targets for EBV-specific recognition (19). These observations suggest that all latent antigens except EBNA 1 are targets for EBV-specific CTL recognition, although a disproportionate number of CTL epitopes are located within the EBNA 3 proteins.

There are two important implications for the overall biology of EBV if subsequent studies confirm that EBNA 1 does not include CTL epitopes. First, the ability of BL cells (which express only EBNA 1) to proliferate in vivo, in spite of a typically normal EBV-specific CTL response (28), is consistent with the observation that there are no CTL epitopes within EBNA 1. In vitro studies have indicated that BL cells are much less susceptible to EBV-specific CTL lysis than LCLs from the same donor (29). Down-regulation of HLA class I alleles (30) and adhesion molecules (e.g., LFA-1 and -3, and ICAM-1) (31) have been implicated as possible mechanisms for this resistance of BL cells to lysis. However, recent observations from this laboratory using peptide epitopes from EBNA 2, 3A, and 3C have demonstrated that downregulation of latent antigen expression is the most critical factor in the nonrecognition of BL cells (Khanna et al., manuscript in preparation).

The lack of detectable CTL epitopes within EBNA 1 has a second important implication in regard to the persistence of EBV in peripheral B cells. A model for the persistence of EBV in B cells has recently been proposed. A feature of this model is the existence of a long-lived, non-replicating, EBNA 1-expressing B cell (32). The observations from the present study provide a mechanism by which these cells can maintain a nonimmunogenic phenotype by not expressing the critical latent proteins needed for CTL recognition.

Of the 20 CTL epitopes localized by recombinant vaccinia, five were defined at the peptide level. In addition to three previously published epitopes (12, 13, 23), in the present study we have defined two new epitopes, one in EBNA 2 and one in EBNA 3C. Although CTL clones specific for nine epitopes recognized both type 1 and type 2 transformants, a significant number of epitopes (7/16 epitopes for which EBV type was determined) were specific for type 1 EBV. Since we have recently shown that the majority of single amino acid substitutions within CTL epitopes result in loss of recognition (33), the common isolation of type 1-specific CTL clones is not unexpected when the degree of latent antigen sequence variation between the two types is compared (7).

The present study has important implications for any future EBV vaccine designed to control IM or EBV-associated tumors. First, CTL epitopes from a spectrum of individuals are distributed throughout most of the latent proteins. Second, >60% of the CTL epitopes are located in regions outside the EBNA/LMP 1 proteins. Both of these considerations suggest that any EBV vaccine based on CTL epitopes designed to provide widespread protection will need to include not only EBNA and LMP sequences but also other regions of the genome expressed during latent infection. However, since CTLs from the majority of donors recognized EBNA 3A and EBNA 3C as target antigens, incorporation of epitopes derived from these proteins into a vaccine may protect the majority of susceptible individuals from IM. The inability of human CTLs to recognize EBNA 1 as a target antigen, often the only latent antigen expressed in BL and NPC, suggests that CTL control of these tumors will not be feasible unless the downregulation of latent antigens can be reversed. The localization of CTL epitopes within LMP, however, raises the possibility of controlling EBV-associated tumors with normal LMP expression (Hodgkin's lymphoma and some NPC) by boosting the CTL response to this antigen.

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Strategies Involved in Developing an Effective Vaccine for EBV-Associated Diseases

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I. INTRODUCTION

Epstein–Barr virus (EBV) is encoded by a linear, double-stranded DNA genome of 172 kb that includes almost 100 identified open reading frames (Baer *et al.*, 1984). The virus maintains a lifelong latent association with B lymphocytes (Yao *et al.*, 1985) and a permissive association with stratified epithelium in the oropharynx (Lemon *et al.*, 1977; Sixbey *et al.*, 1984). The nature of these latent proteins has been studied extensively by exploiting the unique capacity of EBV to immortalize human B lymphocytes *in vitro* (Pope

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et al., 1968). These transformed lymphoblastoid cells (LCLs) express a family of latent proteins including the EBV-induced nuclear antigens (EBNA1, EBNA2, EBNA3, EBNA4, EBNA5, and EBNA6) and latent membrane proteins (LMP1 and LMP2A and -2B). Under certain conditions, LCLs may express the proteins associated with virus replication (Sinclair and Farrell, 1992).

Two major subtypes of EBV have been identified, A type and B type (also known as EBV-1 and EBV-2) (Adldinger et al., 1985; Bornkamm et al., 1980; Dambaugh et al., 1980). EBNA2, EBNA3, EBNA4, and EBNA6 of A-type EBV differ in amino acid sequence from the B-type EBV by 16—47% (Dambaugh et al., 1984; Adldinger et al., 1985; Sample and Kieff, 1990). The biological relevance of these two EBV types has not yet been resolved, but it is well established that A-type EBV can be preferentially isolated from the peripheral saliva of 50% of healthy EBV-seropositive individuals (Sixbey et al., 1989) and B-type virus tends to be detected in a range of clinical conditions associated with immunosuppression (Sixbey et al., 1989; Sculley et al., 1990; Walling et al., 1992; Kyaw et al., 1992). It is not clear at this stage the extent to which this disparity in isolation between different EBV types is a reflection of the poorer growth-transforming capacity of B-type EBV (Rickinson et al., 1987) making it more difficult to detect this strain.

This review will first consider EBV host–virus relationships and then proceed to discuss immune control of EBV infection. These topics will provide a backdrop for an examination of the issues of vaccine development and immunotherapy.

II. HOST–VIRUS RELATIONSHIPS

Latently infected B lymphocytes express only a relatively small number of viral proteins, whereas a wide range of viral genes are expressed in cells undergoing virus replication. It is possible to characterize three distinct forms of EBV latency (latency I, latency II, and latency III) that are distinguished on the basis of expression of EBV latent genes and promoter usage (Hudson et al., (1985; Sample et al., 1986, 1991; Rowe et al., 1986, 1987, 1992; Kerr et al., 1992). These latency patterns or programs form a convenient means of classifying EBV-associated diseases (Table I) and are the basis for vaccine development.

A. Latency I Program

Latency I is characterized by the expression of a single EBV protein, EBNA1 (Rowe et al., 1987), together with a high copy number of small nonpolyadenylated transcripts, EBER1 and EBER2 (Rymo, 1979; Howe

Table 1 EBV Proteins Expressed during Latent and Replicative Infection

EBV gene expression	Latency program			Replicative program
	I	II	III	
EBNA1	+	+	+	+
LMP1	−	+	+	−
LMP2	−	+	+	−
EBNA2–6	−	−	+	−
BHRF1	−	−	+	+
BZLF1	−	−	−	+
Promoter usage in latency	Fp	Fp/Np	Cp/Wp/Np	
References	Rowe et al. (1986, 1987) zur Hausen and Schulte-Holthausen, (1970); Rooney, et al. (1986); Rymo, (1979); Howe and Shu, (1989); Sample et al. (1991); Henderson et al. (1991)	Fahraeus et al. (1988); Brooks et al. (1992); Busson et al. (1992); Khan et al. (1993); Paltesen et al. (1993); Longnecker et al. (1992); Kerr et al. (1992)	Hudson et al. (1985); Dillner et al. (1986a,b); Sample et al. (1986, 1991); Rodgers et al. (1990)	Lennon et al. (1977); Morgan et al. (1979); Sixbey et al. (1984); Takada and Ono (1989); Becker et al. (1991); Sample et al. (1986); D. B. Thomas et al. (1990)

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and Shu, 1989), and the use of the Fp promoter, which allows selective expression of EBNA1 (Sample *et al.*, 1986, 1991). EBNA1 is a DNA-binding protein essential for maintenance of the EBV copy number during cell division (Yates *et al.*, 1984; Sample *et al.*, 1992). The classic features of latency I are exhibited in endemic Burkitts lymphoma (BL) biopsies and in early-passage cell lines derived from these tumors (Rowe *et al.*, 1987). BL displays three reciprocal translocations between chromosome 8, near the site of the c-*myc* locus at 8q24, and either the immunoglobulin heavy chain locus on chromosome 14 (the common translocation seen in up to 80% of tumors) or one of the light chain loci on chromosomes 2 or 22 (the variant translocations (Manolov and Manolova, 1972; Magrath, 1990; Lenoir *et al.*, 1982). Endemic BL is a monoclonal B-lymphoblastic tumor approximately 30 times more common (0.01% prevalence) in areas of Africa and Papua New Guinea where malaria is holoendemic. Such endemic BL cases are invariably pediatric (peak incidence 6–8 years) and 97% are uniformly infected with EBV (zur Hausen and Schulte-Holthausen, 1970; Reedman and Klein, 1973). The link between endemic BL and EBV was enhanced by the discovery that 85% of patients had high anti-early antigen titers, whereas control children, although infected, usually had low or undetectable antibody (G. Henle *et al.*, 1971a).

Explanted BL cells grow continuously in culture, and on serial passage some retain the phenotype of the original biopsy (group I). However, during prolonged culture *in vitro*, many BL cell lines show a dramatic phenotypic drift, with increased expression of B-cell activation antigens and adhesion molecules and the appearance in the culture of clumps of more lymphoblastoid-like cells (group III). As the group III phenotype cells dominate the culture, they frequently lose expression of CD10 and CD77 BL markers, while other LCL-associated markers, such as CD23, CD40, intracellular adhesion molecules, and Bcl-2, are up-regulated (Rooney *et al.*, 1986; Rowe *et al.*, 1987; Henderson *et al.*, 1991). Group II BL cell lines exhibit an intermediate drift toward a group III phenotype. These contrasting features of BL cellular phenotypes are an important tool in understanding the immune escape mechanisms of BL.

It should be borne in mind that, apart from endemic BL, two other forms of this lymphoma are recognized. Both sporadic and an acquired immunodeficiency syndrome–associated BL display the pathological and cytological features of endemic BL but are less commonly EBV positive.

B. Latency II Program

Cells in latency I and II share many features (EBNA1, EBER RNAs, and Fp usage) and differ only with respect to expression of the transmembrane proteins LMP1, LMP2A, and LMP2B. One or more LMP promoters are

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activated in latency II, resulting in expression of LMP1 and/or LMP2A and/or LMP2B (Kerr *et al.*, 1992). LMP1 is essential for transformation of B lymphocytes *in vitro* (Kaye *et al.*, 1993), although LMP2A and -2B are apparently unimportant (Longnecker *et al.*, 1992, 1993). Although LMP1 is associated with induction of the tumorigenic phenotype in rat fibroblasts (Wang *et al.*, 1985), its lack of expression in BL and selective expression in diseases expressing the latency II program (Young *et al.*, 1988; Fahraeus *et al.*, 1988) leave its *in vivo* role in some doubt.

Two clinical conditions exhibit the latency II program, nasopharyngeal carcinoma (NPC) and Hodgkins disease (HD). NPC is a mucosal epithelium tumor of the postnasal space (Ho, 1972) with a high incidence (15–50 × 10⁵/year) in populations from a variety of east Asian and other ethnic backgrounds. It has strong immunogenetic, environmental, and dietary associations (Lu *et al.*, 1990; Liebowitz, 1994). A connection between EBV and NPC cases from diverse geographical origins was initially made on serological grounds (Old *et al.*, 1966), but subsequent studies found that the blood or saliva of NPC and pre-NPC patients contains high titers of immunoglobulin (Ig) A antibodies to a variety of replication-associated antigens (Henle and Henle, 1976; Desgranges *et al.*, 1977; Cheng *et al.*, 1980; Liu *et al.*, 1989; Littler *et al.*, 1990; Ginsburg, 1990; Joab *et al.*, 1991; Yao *et al.*, 1991). *In vivo*, NPC express CD54 (intracellular adhesion molecule type 1 [ICAM-1]) and vascular cell adhesion molecule (Ruco *et al.*, 1994), the B cell marker CD24 (Karran *et al.*, 1995), Ki67 (Zheng *et al.*, 1994), Ki24 (Neidobitek *et al.*, 1992), and class I and possibly class II MHC (Chen *et al.*, 1994).

Although EBV expression has been demonstrated in approximately 50% of cases of HD (Hummel *et al.*, 1992), there remains some doubt about its role in the genesis of this lymphoma. In this malignancy, the transformed, but usually chromosomally normal, Reed–Sternberg (RS) cells are surrounded and outnumbered by untransformed, poorly characterized lymphocytes (mainly CD4+). The RS cells consistently express CD15, CD30, CD74, and human lymphocyte antigen (HLA) class II (Hsu and Hsu, 1994) but many are thought to be HLA class I negative (Poppema and Visser, 1994). The RS cells have been shown to secrete interleukin (IL)-1, IL-5, IL-6, IL-9, tumor necrosis factor-α, macrophage colony-stimulating factor, and transforming growth factor-β and, less frequently, IL-4 and granulocyte colony-stimulating factor. A deregulated cytokine network and/or cell contact–dependent activation may be associated with the HD lesion.

C. Latency III Program

The important features of cells in latency III have been derived from an analysis of LCLs. Cells in latency III are characterized by (1) expression of

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the EBV nuclear proteins EBNA1–6 and LMP1, LMP2A, and LMP2B; (2) expression of cellular genes such as CD23, a ligand for the EBV receptor CD21 (Wang et al., 1987); and (3) a pattern of promoter usage quite distinct from that used in latencies I and II (Sample et al., 1986; Rodgers et al., 1990).

Two EBV-associated diseases best exemplify the latency III program: infectious mononucleosis (IM) and posttransplantation lymphoproliferative disorders (PTLD), the potentially fatal immunoblastic lymphomas in transplant patients. EBV was linked to IM several years after the first description of the virus. Evidence for this association included the spontaneous outgrowth of EBV-infected B lymphocytes from the cultured peripheral blood of patients with acute IM (Pope, 1967) and the coincidence of acute IM and appearance of antibodies to EBV (Niederman et al., 1968; Henle et al., 1968). Over 90% of infants in low per capita income countries seroconvert by the age of 4, whereas 15% of infants in higher socioeconomic strata countries remain EBV-seronegative past the age of 20 (Straus and Fleisher, 1989). The classic signs of IM—swollen posterior cervical lymph nodes, fever, and pharyngitis—are most apparent in adolescents and young adults. In this case, about 50% of seroconversions are associated with clinical symptoms, whereas earlier seroconversion is generally subclinical (Epstein and Achong, 1977). The primary site of virus replication is the pharyngeal epithelium (Gerber et al., 1972; Sixbey et al., 1984), and recirculating B lymphocytes can presumably become latently infected at this primary site of virus replication, thereby generalizing the infection (Pope, 1967).

An association of EBV with PTLD was first noted by Crawford and colleagues (Crawford et al., 1980; Thomas et al., 1991). Histological analysis of PTLD shows a quite complex clonal diversity ranging from polymorphic B-lymphocyte hyperplasia to malignant monoclonal lymphoma. Analysis of the cellular phenotype of PTLD using immunohistochemical techniques has revealed that in most cases the lymphoma cells have phenotypic characteristics similar to LCLs, with high levels of CD23 and intracellular adhesion molecules such as ICAM and lymphocyte functional antigen (LFA-3) (J. A. Thomas et al., 1990; Thomas et al., 1991).

D. A Latency Program of EBV-Infected Resting B Lymphocytes

Following primary infection, EBV persists for life in B lymphocytes and epithelial cells in all healthy seropositive individuals. Details of the cell—virus relationships that are involved in this persistence are not well understood and cannot be readily incorporated into the models of latency previously discussed. Evidence suggests the presence of EBERs, EBNA1, and

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LMP2A transcripts (Tierney *et al.*, 1994) that express the cell surface phenotype CD19 and lack expression of CD23 and CD80 (B7) (Miyashita *et al.*, 1995).

E. Virus Replication Cycle

It is clear from *in vitro* models that all three forms of latency can switch directly into the lytic cycle following activation with phorbol esters, by cross-linking with surface IgM or treatment with calcium inophore (Luka *et al.*, 1979; Takada and Ono, 1989). Alternatively, virus replication can be induced by superinfection of cell lines such as Raji with virus from a latent antigen-defective cell line that complements Raji's defect in replicative gene expression (Biggin *et al.*, 1987). In these reactivation models, nuclear BZLF1 (also known as Zebra) acts as a "lytic switch," launching the productive cycle cascade by activating promoters for itself, BRLF1, and several delayed early proteins (Flemington and Speck, 1990). Studies with these cell lines had previously enabled a division of replicative proteins into the early antigen (EA), membrane antigen (MA), and virus capsid antigen (VCA) complexes. Early immunological studies divided the EA response into restricted (EA-R) and diffuse (EA-D) components. The response to EA-R and EA-D is frequently of diagnostic significance (W. Henle *et al.*, 1971; G. Henle *et al.*, 1971b). VCA is abundantly expressed in cell lines undergoing productive infection, and has polypeptide and glycoprotein components ranging in size from 26 to 200 kDa, with a 143-kDa polypeptide (BNRF1 reading frame) being the major component (Thorley-Lawson *et al.*, 1982).

There are two *in vivo* models available to study EBV replication. Stratified squamous epithelium in immunocompromised individuals with oral hairy leukoplakia (OHL) expresses a range of replicative proteins (Greenspan *et al.*, 1985). Thus in the upper spinous layer in OHL lesions, BRLF1, BMLF1 (pp60 component of EA-D), BHRF1 (p17 component of EA-R), BGLF5 (DNase), and BCLF1 (p150 component of VCA) can all be detected (Becker *et al.*, 1991; Baylis *et al.*, 1991). These results have led to the suggestion that replication is confined to differentiated epithelium, although there is evidence of BZLF1 in the basal layer in this model as well as in the second *in vivo* model, normal tongue mucosal tissue (Becker *et al.*, 1991). Fusion of circulating EBV-positive B cells with oropharyngeal epithelium or uptake of IgA—virus complexes by the epithelium (Sixbey and Yao, 1992) offer alternative explanations for the infection of oral epithelium. It seems likely that these cells release virus as differentiation proceeds and BZLF1 expression increases.

The MA complex is involved in mediating virus binding to the B-lymphocyte receptor CR2 (Nemerow *et al.*, 1985; Tanner *et al.*, 1987) and consists

of at least four major EBV-induced glycoproteins: gp340/300, gp250/200, gp85, and gp78/55 (Mackett *et al.*, 1990). Studies with EBV immune sera demonstrated that neutralization capacity was included in antibody to MA and that MA was a component of the virus envelope (Pearson *et al.*, 1971; North *et al.*, 1982). Neutralizing determinants are included in gp340/220, gp250/200, and gp85 (Hoffman *et al.*, 1980; Qualtiere *et al.*, 1982; Strnad *et al.*, 1982).

III. HUMORAL RESPONSE TO EBV INFECTION

Historically, the humoral response to EBV infection was defined in terms of a set of immunofluorescence assays that quantitatively assessed the antibody response to VCA, MA, EA-R, EA-D, and EBNA. Thus, during the acute phase of IM, patients have high titered IgM and IgG antibodies to VCA and in many cases an IgG response to EA-D, together with an anti-MA response to gp85 rather than toward gp340/220 (Henle *et al.*, 1979). This lack of a response to gp340/220 is important since antibody to this complex is the most potent source of neutralization of the virus. This result appears to bring into question the role of the neutralizing response in recovery from acute infection. Acute IM patients show a pronounced IgM antibody response to auto- and heterophile antigens and a transient IgG response to the EBNA2 protein, whereas the IgG response to EBNA1 is not usually detectable until the convalescent phase (Henle *et al.*, 1974, 1987). During the convalescent period, the IgM response falls while the IgG response to VCA and EBNA1 plateaus at a reduced level without any obvious protective role. It has also been suggested that antibody to gp340/220 binds to productively infected cells, rendering them susceptible to antibody-dependent cellular cytotoxicity (ACDD)-mediated lysis (Patarroyo *et al.*, 1980).

It is important to consider the role of the neutralizing response seen during acute IM. There is certainly some doubt about a true viremia, although high levels of infectious virus are seen in saliva. Whereas the emerging IgG response to gp340/220 has the potential to render gp340/220-positive cells susceptible to ADCC, the kinetics of this response in IM patients argues against any primary role in limiting the spread of infection (Pearson *et al.*, 1979).

IV. CTL RESPONSE TO EBV INFECTION

Significantly increased shedding of EBV in the oropharynx of immunosuppressed individuals provides evidence in support of an important role for T

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cells in controlling EBV infection. This control may be exercised at the level of replicative infection in the oropharynx or at the level of B cells expressing latent antigens. Although most contemporary reports have focused on the importance of cytotoxic T lymphocyte (CTL) control mediated through recognition of latent antigens (see later), it should not be assumed that control through this effector mechanism is the most important. For example, early studies demonstrated that T cells inhibit the proliferation of EBV-infected B cells (Thorley-Lawson et al., 1977; Thorley-Lawson, 1980) and that this inhibition appears to be related to the release of γ-interferon by CD4+ T cells (Hasler et al., 1983; Anderson et al., 1983). More recent studies using a related system have verified a role for T cells in inhibiting cells expressing replicative antigens, including gp340 (Bejarano et al., 1988, 1990). However, in spite of this evidence of the importance of T-cell control of replicative antigens, the experimental systems available for studying the CTL response to latent antigens have ensured a far greater understanding of the role of this effector mechanism in primary EBV infection, in long-term healthy carriers, and in those suffering from EBV-associated disease.

A. CTL Response during Primary Infection

The effector mechanisms involved in recovery from primary EBV infection have been inferred almost exclusively by studying the T-cell response during acute IM. It must be borne in mind that, for the majority of individuals, primary EBV infection does not result in clinical disease. Thus there is a degree of uncertainty (both quantitative and qualitative) about the extent to which the cellular events seen in acute IM differ from those seen during a subclinical EBV infection.

The acute phase of IM is characterized hematologically by a marked CD8+ lymphocytosis, although both CD8+ and CD4+ T cells express activation markers such as HLA-DR (Tomkinson et al., 1989). Recent evidence has shown that both populations exhibit an increased percentage of cells expressing CD45RO (Miyawaki et al., 1991) antigen, which is now considered a sensitive marker for memory T cells. At this stage it is not clear how the primary EBV infection is driving this activated phenotype.

The specificity and function of these activated T cells have been difficult to assess. Since infected B cells in acute IM exhibit latency III, and EBV proteins and oropharyngeal epithelial cells express replicative antigens, an effective CTL response may be directed through any or all of these antigens. Despite early reports in which there was confusion over the apparent nonrestricted nature of the CTL response in acute IM (Svedmyr and Jondal, 1975; Royston et al., 1975), it has been subsequently possible to demonstrate a specific and restricted component of the primary T-cell response together with an allospecific response (Gaston et al., 1983). This specific response is

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apparently a composite response and includes CD8+ (Strang and Rickinson, 1987; Tomkinson *et al.*, 1989), CD4+ (Misko *et al.*, manuscript in preparation), and CD4+ and CD8+ CTLs (Jacobs *et al.*, manuscript in preparation). The target antigens recognized by these specific CTLs include both latent and replicative antigens. It will be important in developing a successful vaccine strategy against IM to establish the respective roles of these effector cells in defining the progress of the primary infection toward either silent EBV seroconversion or acute disease.

B. CTL Response in Healthy Virus Carriers

There is considerable evidence for the existence of a potent EBV-specific memory T-cell population in all healthy virus carriers. This response is apparently directed toward epitopes within the latent EBV antigens rather than epitopes within the replicative proteins. The basic observation illustrating the importance of CTL control grew out of studies comparing the course of EBV transformation of lymphocytes from healthy immune and nonimmune individuals. Thus when adult donor lymphocytes (including T cells) are exposed to the virus and placed in culture, the proliferation of virus-infected B cells, which occurs within the first 2 weeks postinfection, is followed by a complete regression of growth brought about by CTLs reactivated *in vitro* (Moss *et al.*, 1978). In contrast, the proliferation of lymphocytes from nonimmune individuals is not impeded by EBV-specific CTLs. The strict cell concentration dependence of regression (regression occurs at high and not low cell concentration) has become a very convenient semi-quantitative assay for EBV-specific CTLs (Moss *et al.*, 1978). The regression phenomenon is mainly dependent upon the presence of CD8+ CTLs (Moss *et al.*, 1978; Crawford *et al.*, 1983), although the regression endpoint may be influenced by natural killer cell activity (Masucci *et al.*, 1983). Regression may also be assessed by ^{3}H-thymidine incorporation (Thorley-Lawson *et al.*, 1977; Schooley *et al.*, 1981, 1984).

The peptide specificity of CTL clones in regressing cultures from immune individuals has now been analyzed in considerable detail and has revealed the existence of CTL epitopes within eight of the nine latent antigens. The key observation in the identification of target antigens was that, in certain donors, it was possible to exploit the allelic polymorphism in the EBNA proteins between A-type and B-type EBV by isolating A-type–specific CTL clones (Moss *et al.*, 1988). These clones provided an opportunity to define CTL epitopes by screening selected EBNA peptides for reactivity on B-type transformants (Burrows *et al.*, 1990). The peptide epitopes thus far defined are summarized in Table II. It is now clear that the majority of CTL epitopes are encoded within EBNAs 3, 4, and 6. It should be emphasized that this apparent focusing of CTL response through some latent antigens may be a

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Table II   Defined Peptide Epitopes of EBV

EBV antigen	Peptide epitope	HLA restriction	EBV type	Reference
EBNA1	TSLYNLRRGTALA[a]	HLA-DR1	A&B	Moss et al. (1994)
EBNA2	DTPLPLTIF	HLA-B51/A2	A	Schmidt et al. (1991)
EBNA3	FLRGRAYGL	HLA-B8	A	Burrows et al. (1992)
	QAKWRLQTL	HLA-B8	A	Burrows et al. (1994)
	SVRDRLARL	HLA-A2	A&B	Burrows et al. (1994)
	YPLHEQHGM	HLA-B35	A	Burrows et al. (1994)
	HLAAQGMAY	HLA?	A	Burrows et al. (1994)
	RPPIFIRRL	HLA-B7	A	Hill et al. (1995)
EBNA4	IVTDFSVIK	HLA-A11	A	Gavioli et al. (1993)
	AVFDRKSDAK	HLA-A11	A	Gavioli et al. (1993)
	AVLLHEESM	HLA-B35	A	Khanna et al. (unpublished)
EBNA6	EENLLDFVRF	HLA-B44	A&B	Burrows et al. (1990)
	KEHVIQNAF	HLA-B44	A	Khanna et al. (1992)
	RRIYDLIEL	HLA-B27	A	Brooks et al. (1993)
	QPRAPIRPI	HLA-B7	A&B	Hill et al. (1995)
LMP2A	CLGGLLTMV	HLA-A2	A&B	Lee et al. (1993)
	IYLFWLAAI	HLA-A23	A&B	Khanna et al. (unpublished)

[a] Sequence recognized by CTL but apparently not processed.

reflection of the choice of volunteers in these studies, who were almost exclusively Caucasian. It is possible this response may more frequently include other latent antigens as these studies are extended to include other ethnic backgrounds. It is interesting that HLA class I alleles such as A2, B7, B8, B35, B40, and B51 were each shown to present more than one distinct CTL epitope derived from different latent antigens (Khanna et al., 1992).

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Although some of the epitopes defined thus far are conserved in both A-type and B-type transformants, a significant number are specific for A-type EBV (Table II). Since it is well established that the majority of single amino acid substitutions within CTL epitopes can result in loss of recognition (Burrows *et al.*, 1992), the common isolation of A-type–specific CTL epitopes is not unexpected when the degree of latent antigen sequence variation between the two types is compared (Sample and Kieff, 1990).

The question of mutations at the site of CTL epitopes is important to consider in relation to EBV vaccines. Studies on a panel of A-type EBV isolates derived from different geographical locations indicated that two CTL epitope sequences (HLA-b8–restricted epitope FLRGRAYGL and HLA-B44–restricted epitope EENLLDFVRF) were widely conserved. However, recently a polymorphism within the HLA-A11–restricted CTL epitope, IVTDFSVIK, derived from EBNA4, has been reported (de Campos-Lima *et al.*, 1993, 1994). Although the epitope sequence is conserved in most A-type strains worldwide, all of the A-type strains isolated from individuals from Southeast Asia and Papua New Guinea (coastal region), where the HLA-A11 allele frequency is very high, consistently carried an altered sequence with specific mutations in one of the anchor residues. These mutant peptide sequences do not bind to HLA-A11 molecules and are thus not recognized by HLA-A11–restricted EBV-specific CTLs. This study and recent observations from our laboratory (unpublished) raise the possibility that mutations at the site of CTL epitopes may be more common than had been previously suspected.

One of the most interesting facets of this body of work is the response to EBNA1 protein. This consideration assumes particular importance in the present context, since a successful vaccine that included this antigen might encompass all EBV-associated diseases. Although earlier studies failed to demonstrate CTL epitopes within EBNA1, recent studies have identified a major histocompatibility complex (MHC) class II–restricted peptide sequence that is recognized by CTLs after exogenous sensitization of autologous or HLA-DR1–positive B cells (Moss *et al.*, 1994). One of the most important features of this epitope is that EBV-transformed LCLs and BL cell lines are unable to process and present this epitope endogenously. It is interesting to note that this epitope is included in the EBNA1 DNA-binding region, which may limit transport of the epitope into the class II processing compartment, thus blocking effective processing through this pathway.

C. T-Cell–Mediated Control of EBV-Associated Tumors

There is increasing evidence for the direct involvement of cell-mediated immune mechanisms in the control of human tumors. This concept is based

mainly on the assumption that, like virus-infected cells, tumor cells can present on their surfaces certain tumor-specific antigens as short peptides in association with MHC molecules that can be recognized by CTLs. Defects in this process represent a potential risk, since it is an essential link in CTL-mediated immune surveillance. The potency of the CTL response is frequently such that outgrowth of tumor cells in the immunocompetent host is only achieved through some form of immune evasion. EBV-associated tumors such as BL and NPC are classic examples in this category (Moss et al., 1983, 1994; Rooney et al., 1985b). In contrast, outgrowth of PTLD is modulated by the immunocompetence of the host. It is generally assumed that the sensitivity of these tumors to EBV-specific CTLs is similar to that for LCLs since the phenotype of each is identical. It is well established that relaxation of immunosuppressive therapy (with a presumptive rise in the level of EBV-specific CTLs) can lead to regression of these lymphomas.

Endemic BL provides an ideal model for understanding the mechanism(s) of immune evasion by EBV-associated tumors, primarily because of the ease with which cell lines can be established from this tumor. It is now well established that group I BL cell lines are highly resistant to virus-specific CTL lysis and are poorly immunogenic in their ability to stimulate an alloresponse (Rooney et al., 1986). Studies by Rooney and colleagues identified a number of BL patients who retain detectable EBV-specific T-cell surveillance, indicating that CTL dysfunction is an unlikely cause of the outgrowth of these tumors in vivo (Rooney et al., 1985a). However, when matched sets of LCLs and BL cell lines from the same patient are compared, the group I BL cells are not lysed in vitro by polyclonal EBV-specific T-cell lines or CTL clones (Khanna et al., 1993; Rooney et al., 1985b). The lack of CTL lysis of BL cells is not due to any inherent resistance of these targets to cytolysis, since such lines are killed by both allospecific CTL clones (Torsteinsdottir et al., 1986) and non–MHC-restricted lymphokine-activated killer (LAK) cells (Misko et al., 1990). It is interesting to note that, as EBV-positive BL cell lines drift from a group I to a group III phenotype, they show increased susceptibility to EBV-specific CTL recognition (Rooney et al., 1985b). Next we discuss three different possible mechanisms that have been proposed to explain the immune evasion by EBV-associated malignancies.

I. DOWN-REGULATED EXPRESSION OF ADHESION MOLECULES

The role of down-regulated expression of adhesion molecules in immune evasion was revealed from the study of phenotypically stable group I BL cell lines (Gregory et al., 1988). An absence or very low expression of the cellular adhesion molecules such as LFA-1, LFA-3, and ICAM-1 was consistently seen in BL cell lines. This is illustrated in Table III by the results of a

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Table III Phenotypic Characteristics of Groups I and III BL Cell Lines

Cell line	Phenotypic group	Growth pattern	Adhesion molecule expression (MFI)[a]		TAP-1 and TAP-2 expression[b]		CTL susceptibility	
BL29	I	Single cell	LFA-1α	14.37	TAP-1	−	Allospecific	
			LFA-1β	15.25	TAP-2	−	CTL lysis	+++
			LFA-3	17.21				
			ICAM-1	3.35			Virus-specific	
			ICAM-2	127.34			CTL lysis	−
BL18	III	Large clumps	LFA-1α	117.89	TAP-1	+++	Allospecific	
			LFA-1β	126.38	TAP-2	+++	CTL lysis	+++
			LFA-3	114.19				
			ICAM-1	90.74			Virus-specific	
			ICAM-2	132.42			CTL lysis	+++

[a] MFI, mean fluorescence intensity.
[b] Tap-1 and Tap-2 expression in these cell lines was analyzed at mRNA and protein levels.

fluorescence activated cell sorter (FACS) analysis of group I (BL29) and group III (BL18) cells after immunofluorescence staining with monoclonal antibodies specific for these molecules. These differences are reflected in the differing growth patterns of group I and group III cells; for example, BL29 cells grow as a single cell suspension rather than in clumps because the molecules mediating homotypic adhesion, namely LFA-1 and ICAM-1, are at very low levels. More importantly, low expression of ICAM-1 and LFA-3 on the group I BL cell surface is reflected functionally by the inability of such cells to form conjugates with activated T cells in short-term *in vitro* assays (Gregory et al., 1988). These characteristics are not unique to EBV-positive BL, since cell lines established from EBV-negative, sporadic BL show the same phenotype. However, studies have demonstrated that, whereas the virus-specific CTL recognition of LCLs is dependent on an intact LFA-3–CD2 pathway, BL cell lysis could be achieved by peptide sensitization and recognition through the LFA-1–ICAM-2-pathway (Khanna et al., 1993). Because there is consistently high expression of ICAM-2 on all BL cell lines (Table III), it appears that down-regulation of LFA-1, LFA-3 and/or ICAM-1 expression on BL cells does not provide an absolute barrier to tumor cell recognition by virus-specific CTLs.

2. LIMITED VIRAL GENE EXPRESSION

The viral phenotype of BL cells is likely to be a very important factor in reducing tumor susceptibility to EBV-specific CTL surveillance. EBNA1 is

the only detectable latent protein in group I BL cells, with all other EBNAs and LMPs down-regulated (Rowe et al., 1987). It has generally been considered that EBNA1 does not include epitopes that are presented for CTL recognition by EBV-infected cells. Thus it is not hard to see the advantage that this highly restricted form of EBV latency offers BL cells in terms of immune evasion, since EBNA2–6 and the LMPs, which constitute the dominant targets of virus-specific CTL recognition (Khanna et al., 1992; Murray et al., 1992), are not expressed. It seems unlikely that restricted EBV gene expression in BL cells is a result of an immune pressure *in vivo*, that is, that down-regulation of latent protein expression is a key selective step in the pathogenesis of BL. Indeed, the latency program in BL may reflect that of its progenitor B lymphocyte, rather than a unique feature of a malignant cell clone selected for its ability to evade EBV-specific CTL recognition.

In contrast, it is surprising that the expression of the highly immunogenic antigens LMP1 and LMP2 in NPC and HD does not result in rejection of these tumors *in vivo*. A possible explanation is provided by a murine model that demonstrated that the *LMP1* gene from NPC, when expressed in mouse carcinoma cells, was completely nonimmunogenic, whereas the *LMP1* gene from the B95.8 cell line was highly immunogenic (Trivedi et al., 1994). Interestingly, LMP1 encoded by NPC isolates shows numerous amino acid changes as compared to the B95.8 sequence (Hu et al.,1991). More recently, similar amino acid changes in the *LMP1* gene have also been reported in EBV isolated from HD (Knecht et al., 1993). These observations strongly suggest that mutations in the LMP1 sequence might render tumor cells nonimmunogenic for CTLs. Although a number of CTL clones specific for LMP1 have been isolated from healthy EBV-immune individuals (Khanna et al., 1992), the peptide specificity for these clones has not yet been defined. It will be interesting to see whether the mutations within the LMP1 sequence of NPC isolates are encoded within epitope sequences that are relevant for CTL recognition. These observations therefore provide an interesting focus for future work intended to delineate the role of the EBV-specific CTL response in the control of NPC and HD.

3. DOWN-REGULATED EXPRESSION OF ANTIGEN-PROCESSING GENES

Well before the molecular identity of EBV target antigens was resolved, Rowe and colleagues proposed that the insensitivity of EBV-positive BL cell lines to virus-specific CTLs might reflect a cellular defect in the ability to process and present latent antigens to the T-cell system, rather than absence of the antigens *per se* (Rowe et al., 1987). Given what we now know about processing of endogenously synthesized proteins to small peptide fragments and their presentation on the cell surface as a complex with HLA class I

antigens, it is clear that endogenous antigens have the potential to provide target epitopes for a specific CTL response. These peptides are transported into the endoplasmic reticulum by a family of transporters associated with antigen processing (TAP-1 and TAP-2), where these peptides associate with MHC class I molecules.

It has been clear for many years that BL biopsy cells and derived BL cell lines do express HLA class I antigens on the cell membrane, and the levels of HLA antigens expressed on the group I BL cell surface are sufficient to sensitize these cells to lysis by appropriate allospecific CTL lines (Torsteinsdottir et al., 1986). However, recent studies have raised the possibility of loss of MHC conformation as a result of a defective endogenous peptide loading of MHC molecules compared to the autologous LCLs (Khanna et al., 1994). These observations have subsequently been confirmed by immunoprecipitation and isoelectric focusing (Rowe et al., 1995). Furthermore, recent results have demonstrated that the class I antigen processing pathway is very much less active in BL cells than in LCLs (Khanna et al., 1994; Rowe et al., 1995). This is reflected in very low expression of the transporter proteins TAP-1 and TAP-2 in group I BL cell lines. Deficiencies in this antigen-processing pathway in laboratory-generated antigen-processing mutant cell lines (such as RMA-S and T2) are generally associated with reduced stability and surface expression of MHC class I molecules (Ljunggren et al., 1990; Momburg et al., 1992). This reduction is apparently due to a deficient supply of peptide epitopes into the endoplasmic reticulum for MHC stabilization. However, in contrast to the RMA-S and T2 cell lines, in which the TAP genes are deleted, BL cell lines show a transcriptional deficiency with significantly reduced levels of TAP-1 and TAP-2 mRNA and protein expression (Khanna et al., 1994). At present nothing is known about the status of TAP genes in undifferentiated NPC and HD. It will be important to determine whether antigen-processing capacity in these tumor cell backgrounds contributes in any way to their ability to evade an effective EBV-specific CTL response.

V. DEVELOPING A VACCINE FOR EBV

During the past 10 years, the understanding of immunological control of EBV-associated diseases has proceeded to the point where serious consideration of the development of a vaccine is timely. Given the oncogenic potential of the virus and its narrow host range, it is appropriate that these considerations proceed on two parallel paths. In the first place, it is important to begin human trials using existing technology as soon as possible, and several such trials are described in Section VI. It is equally important to be

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open to exploit new approaches to vaccine design that draw on experiences with human trials on related viruses and on animal models of EBV-associated disease. In particular, a number of human vaccine trials involving human cytomegalovirus (HCMV) have already been conducted, and these are summarized here together with several animal models that are based on either EBV or other gamma herpesviruses that, in some cases, include both lytic and latent components in their infection strategy.

A. Lessons from Another Herpesvirus

HCMV is highly prevalent throughout the world, particularly in individuals whose immune systems are compromised, such as transplant patients (Stagno et al., 1982). These clinical observations have provided considerable impetus for the development of an effective vaccine. The successful demonstration of adoptive transfer of CMV-specific CD8+ CTL in humans (Riddell et al., 1992) and mice (Jonjic et al., 1988), together with a lack of success of passive immunization with heterologous human antibody (Boland et al., 1993), makes it likely that CTLs are the main protective mechanism. There is now considerable experience in the use of live attenuated vaccines to HCMV using normal adults and transplant volunteers. The first inoculations into humans involved a laboratory-adapted strain from adenoidal tissue that is referred to as AD-169 (Elek and Stern, 1974; Neff et al., 1979). These trials demonstrated that vaccination induced a significant neutralizing and complement-fixing response and was not associated with significant sequelae. Subsequent vaccine studies have involved the Towne strain, which was originally isolated from a congenitally infected infant and subsequently passaged in WI-38 human diploid fibroblasts (Plotkin et al., 1976; Plotkin et al., 1991; Balfour et al., 1985). This strain of HCMV is antigenically related to other HCMV strains, induces a specific CTL response (Quinnan et al., 1984), exhibits greater release of extracellular virions and thermostability than wild-type strains of the virus, and does not establish latency. Several trials have been carried out in renal transplant patients using the Towne strain. The most important aspect of these trials is that vaccinated HCMV-negative individuals were significantly protected from clinical disease related to the virus following organ grafting from HCMV-positive individuals.

Another approach that has proved successful in protecting against CMV challenge has recently been developed. Mice vaccinated with a CTL peptide epitope from murine CMV (from the immediate EA1) in an appropriate adjuvant formulation (Montanide ISA 720), which includes tetanus toxoid helper activity, were protected from subsequent challenge (Scalzo et al., 1995). One of the more interesting aspects of this body of work is that protection appeared to be relatively independent of peptide concentration.

This result is important since it suggests that it might be possible to translate this vaccination strategy to humans. Indeed, this approach formed the basis of a peptide-based vaccine trial currently being undertaken in this laboratory (see VI, A).

B. Animal Models

Several animal models using EBV or related viruses have been developed that may be useful in developing vaccine strategies. These models include EBV-induced lymphomas in cottontop tamarins and severe combined immunodeficiency (SCID) mice, and a parallel murine gamma herpesvirus, herpesvirus-68 (MHV-68).

The cottontop tamarin (*Saguinus oedipus oedipus*) is a model that parallels EBV in the development of multiple B-cell lymphomas in 100% of unprotected animals within 3 weeks after intraperitoneal inoculation with high-titered EBV (Finerty et al., 1992). Purified or recombinant gp340 inoculated within various adjuvants, such as liposomes, muramyl dipeptide (MDP), immunostimulatory complexes (ISCOMS), and alum, or delivered by live vectors such as vaccinia and adenovirus, have demonstrated variable induction of virus-neutralizing gp340 antibodies and in some cases have shown protection from tumor development after high-dose EBV challenge (Epstein et al., 1985; Finerty et al., 1992). This result brings into question the mechanism of protection operating in these various vaccine preparations, since immunochemical assays and immunoblotting indicate that latent rather than replicative antigens (including gp340) are expressed in these tumors (Young et al., 1989). Indeed, there have been suggestions of a cell-mediated mechanism (Young et al., 1989). The results to date highlight the importance of developing *in vitro* assays to define the mechanism for protection in this model (Ulaeto et al., 1988). Immune control over virus replication at sites of productive infection has not been systematically studied (Ulaeto et al., 1988). With further refinement and identification of the exact cellular mechanism and antigens involved, the EBV-induced tumor model in the cottontop tamarin provides an experimental analogue to PTLD.

SCID mice lack functional T and B lymphocytes as a result of a mutation affecting the recombinase systems (Mosier et al., 1988). Injection of human peripheral blood mononuclear cells (PBMCs) from seropositive donors into these mice results in the development of heterologous EBV-positive B-cell lymphomas within 6–10 weeks (Mosier et al., 1988; Veronese et al., 1992). The transplanted human PBMCs remain functional and active for 6 months, although some doubt exists as to the maintenance of CTLs over this time frame (Mosier et al., 1988; Boyle et al., 1993). These tumors contain large, noncleaved lymphoid cells with variable plasmacytoid morphology similar

to PTLD and are transplantable into naïve SCID mice (Veronese *et al.*, 1994). Analysis of EBV gene expression in these tumors reveals that they exhibit the features of latency III.

It is particularly relevant that adoptive transfer of HLA-identical CD8+ EBV-specific CTLs significantly delayed or prevented engraftment of these lymphomas (Boyle *et al.*, 1993). This result is reminiscent of the human studies showing regression of immunoblastic lymphomas following adoptive transfer of EBV-specific CTLs (Rooney *et al.*, 1995). There is evidence that human cytokines IL-6, IL-10, and IL-10 receptors are produced by these tumors and may be involved either directly or indirectly (e.g., by prevention of programmed cell death and induction of proliferation) in tumor development (Veronesi *et al.*, 1994; Veronese *et al.*, 1994). There are several curious features of this model that warrant comment. First, there is an absolute requirement for the inclusion of T cells (most likely CD4+) in the PBMCs for the progression of these tumors (Veronese *et al.*, 1992; Veronese *et al.*, 1994). Second, pretreatment of SCID mice with cyclosporin A prevented, rather than enhanced, formation of these tumors (Boyle *et al.*, 1993). In terms of EBV vaccination strategies, this model could be used to study the selective blockage of specific cytokines by CD4+ cells and their role in the activation of EBV-specific CD8+ CTLs.

A naturally occurring herpesvirus of wild rodents, MHV-68 is an interesting model of both lytic and latent EBV infection. This virus was originally isolated from bank voles (*Clethriononnys glareolus*) in Czechoslovakia and has been reclassified in the gamma herpesvirus family on the basis of electron microscopy, antigenic and cytopathic effects on cell lines, genomic arrangement and homology, and latency identified in B lymphocytes (Efstathiou *et al.*, 1990b; Sunil-Chandra *et al.*, 1992b). As yet, the transforming capacity of MHV-68 *in vitro* has not been reported. It is likely that this will be a prerequisite to defining the analogy between the MHV-68 and EBV latent antigens and their role as target antigens for specific CTLs. MHV-68 produces a consistent respiratory infection via the intranasal route, presumed to be the natural mode of infection, and is spread to various tissues within the body via the blood (Sunil-Chandra *et al.*, 1992a). MHV-68 mimics EBV by inducing both asymptomatic and symptomatic acute infections (Sunil-Chandra *et al.*, 1992a) after intranasal administration in BALB/c mice.

The virus infection resembles EBV in a number of other important characteristics. First, MHV-68 replicates in epithelial tissue, including cell lines, and is apparently latent in a subset of B lymphocytes (Svobodova *et al.*, 1982; Sunil-Chandra *et al.*, 1992b). Second, there is homology between some EBV and MHV-68 genes (Efstathiou *et al.*, 1990b). Third, there is an analogy between lymphoid infiltrates seen in the spleens and lungs of MHV-68—infected mice and the corresponding infiltrates seen in IM (Nash

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and Sunil-Chandra, 1994). Indeed, B-cell lymphomas frequently arise in chronically infected animals (Ehtisham *et al.*, 1993). Fourth, it has been established that CD8+ cells are required for recovery from an acute infection (Ehtisham *et al.*, 1993).

MHV-68 could provide an attractive model to stimulate the EBV lymphomas that develop in humans after immunosuppression. It should be possible to define the conditions under which adoptive transfer of specific cell populations induces regression of these tumors in a manner similar to that reported previously in humans (Rooney *et al.*, 1995). The virus offers an opportunity to study the acute lytic phase of a gamma herpesvirus infection because it readily replicates in epithelial cells *in vivo* and *in vitro* (Svobodova *et al.*, 1982; Sunil-Chandra *et al.*, 1992a; Stewart *et al.*, 1994). The MHV-68 model would also provide a feasible system for defining the role of cell-mediated regulation of the lytic phase of infection.

These three animal systems provide opportunities to evaluate and manipulate immunological strategies for vaccine design. Cottontop tamarins are rare and costly animals, and careful experimental planning will need to be exercised. The advantage of the mouse models is that the animals are inexpensive and the immunology and genetic strains are well defined (Efstathiou *et al.*, 1990a). No model using EBV is ideal, but dissection of the mechanisms regulating the disease states induced in these model systems provides the basis for trial of specific EBV vaccines.

VI. VACCINES AND IMMUNOTHERAPY FOR EBV-ASSOCIATED DISEASES

It is unlikely that a single vaccine that is applicable to all EBV-associated diseases will be developed in the near future. Given the variety of potential EBV targets in latency III diseases and the problems of immune recognition of latency II and latency I diseases previously discussed, vaccines against IM and PTLD would seem to offer the best opportunity for early development. Given this caveat, however, early human trials aimed at BL, NPC, or HD deserve serious consideration. These trials might not be restricted to therapeutic or preventive vaccines but may well encompass new developments in immunotherapy. The following section will discuss some of these issues.

A. Vaccines for Latency III Diseases

The first trial of an EBV vaccine has been reported by Gu *et al.* (1993). The immunogen was a live recombinant vaccinia virus (strain *Tien Tan*) encoding

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the EBV MA BNLF1 (gp340/220). The trial was based on 19 children (9 were immunized with the recombinant vaccinia and 10 served as controls) with no detectable antibody to EBV antigens. All children in the test group developed a neutralizing response to EBV. Sixteen months after vaccination, anti-VCA antibody was detectable in all of those in the control group, whereas 6 of 9 children who had received the recombinant vaccinia remained anti-VCA negative. This result is important and implies that the anti-MA response may have absolutely blocked EBV infection (based on a lack of antibody to VCA) and induced sterile immunity (at least in the short term).

Despite the promising results described in this study, there remains some doubt about the widespread acceptability of using recombinant vaccinia. This factor has led to a search for a subunit vaccine that would have the advantage of being chemically defined and free of any genomic material with replicative capacity. Considerable interest has revolved about the use of a gp340 subunit vaccine based on the early promise of a tamarin model. This vaccine, as with all subunit vaccines for use in humans, is limited by the choice of adjuvant that will be acceptable for human use. At present this choice is limited to alum, which, although efficient at inducing a humoral response, is relatively inefficient at inducing a CTL response, which is the presumed means of blocking the lymphoproliferative tumors in tamarins. It should be pointed out that some caution must be exercised in extrapolating the results in tamarins to those one might expect in humans. Following EBV infection, tamarins do not develop an acute IM-like syndrome, nor do they develop a long-term persistent infection. The results, however, justify continued research into a subunit MA vaccine.

EBV vaccines based on CTL induction aim at reducing morbidity rather than preventing infection. The importance of the CTL response in controlling EBV-associated disease was unequivocally demonstrated recently in the case of PTLD. Unirradiated donor leukocytes (Papadopoulos et al., 1994) or in vitro cultured EBV-stimulated uncloned CTL lines (Rooney et al., 1995) successfully resolved these lymphomas. Although it might be possible to apply this curative approach in this specialized clinical situation, adoptive transfer of specific CTLs is unlikely to find widespread use. These trials, however, established the important principle that specific CTLs are capable of recognizing these tumors and give impetus to efforts at designing a CTL-based vaccine against latency III diseases. A trial in this laboratory is at present being conducted. This trial aims to induce a specific CTL response in healthy EBV-seronegative, HLA-B8 volunteers following vaccination with the peptide epitope FLRGRAYGL (sequence from EBNA3) incorporated into an water-in-oil emulsion (Montanide ISA 720) in the presence of tetanus toxoid. There are several inherent advantages of this particular experimental vaccine: (1) the epitope is conserved in A-type EBV isolates from

Western communities, (2) HLA-B8 is common and essentially non-polymorphic, and (3) most seropositive HLA-B8 individuals respond to this epitope. The vaccine formulation is based on the principles established with the murine CMV model discussed previously in which protection from challenge was demonstrated following administration of a single immunizing dose of a peptide (Scalzo et al., 1995). This phase 1 trial aims to demonstrate the safety of this vaccine in humans as well as to determine its ability to induce a peptide-specific response. The response will be monitored using the regression assay and the isolation of peptide-specific clones. If successful, early recipients of this vaccine might be young children undergoing organ transplant. Many such children are EBV seronegative and are likely to receive blood and tissue from adults latently infected with the virus, and therefore are at considerable risk of PTLD.

Obviously, one of the major obstacles of using multiple peptide epitopes to vaccinate a community is HLA polymorphism, since each HLA class I allele presents a different epitope. One approach might be to mix defined epitopes into a single vaccine. It is estimated that the peptide epitopes defined to date span about 40% of the Caucasian population. It should be borne in mind, however, that as mentioned earlier, there is increasing evidence of population heterogeneity at the site of EBV peptide epitopes, particularly in relation to strains isolated from Papua New Guinea. Fortunately, at present, there is unlikely to be a significant demand for a vaccine to latency I and III diseases from this area of the world. This estimate, however, does not take into account the question of the requirement for protection against both EBV types. As mentioned previously, although A type appears to be the dominant strain in healthy individuals, the observation that B-type EBV can frequently be isolated from immunocompromised individuals suggests that infection with this strain is widespread. It is possible that a successful vaccine may need to incorporate epitopes from A-type as well as B-type EBV since in many instances reactivity to these epitopes is not cross-reactive (Table II). Thus a vaccine based on CTL epitopes that incorporates peptides from both EBV strains and peptides that bind to a wide range of HLA types is likely to be very complex and may pose significant problems with regulatory authorities. Another approach might be to make use of a recent technical advance (Thomson et al., 1995) in which minimal EBV CTL epitopes were fused together to construct a recombinant or synthetic polyepitope protein in which defined epitopes are encoded in a continuous string (rather than in their natural context within a protein). Such a "polytope" vaccine may find application within a conventional vector suitable for humans or injected directly as purified DNA. It has recently been demonstrated that epitopes specified by such a string are indeed presented on the cell surface for recognition by CTLs (Thomson et al., 1995).

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B. Immunological Intervention in Latency I/II Tumors

EBV vaccines directed against latency III diseases and potential vaccines against EBV-associated tumors (latency I and II) are conceptually quite distinct. In general, these tumors have evoked a variety of escape mechanisms that have enabled them to expand in the face of an existing EBV-specific response. A prophylactic vaccine that achieves lifelong EBV sterile immunity represents the ideal goal for controlling these tumors before they develop. However, given their comparative rarity, this approach is unlikely to be cost effective. The only exception may be NPC in specific regions of China where 1–2% of the population will develop this tumor. Immunological strategies against EBV-associated tumors are thus likely to be therapeutic and could exploit the presence of EBV in the tumor cells or could focus on non–EBV-encoded antigens.

1. EXPLOITING THE PRESENCE OF EBV

An attractive strategy for elimination of EBV-associated tumors would be one that turns latency I/II tumors into targets for the existing EBV-specific immunity. In the case of BL, phenotypic drift is frequently seen after *in vitro* culture and is associated with changes in both cellular and viral gene expression. These changes result in a dramatic shift in the tumors' susceptibility to EBV-specific CTLs (Rooney *et al.*, 1985b). Since all three immunologically relevant parameters (i.e., HLA antigens, adhesion molecules, and viral antigens) tend to show coordinate up-regulation in this system, it is very difficult to isolate any one variable and determine its individual importance. However, it has been possible to define the effect on CTL recognition of restoring these functions in isolation.

The individual importance of class I allele down-regulation is perhaps best exemplified with the WW1BL cell line, which is characterized by (1) complete allele-selective suppression of HLA-A11 expression (Torsteinsdottir *et al.*, 1988), (2) expression of LFA-1 and ICAM-1 but LFA-3 negativity (Gregory *et al.*, 1988), and (3) expression of a full complement of EBV latent genes. Stable transduction of the HLA-A11 gene alone restored CTL recognition of this cell line (Torsteinsdottir *et al.*, 1988). However, it is clear in this case that increased expression of this allele alone is not sufficient to break down the barrier to recognition of group I/II BL cell lines displaying down-regulated expression of EBV latent antigen.

Reversal of the down-regulated expression of TAP-1 and TAP-2 proteins may offer the potential to increase immunogenecity and to restore CTL recognition of BL cells. This has been well illustrated recently in a group I BL cell line that was resistant to specific CTL recognition. In this case, trans-

location of a peptide epitope directly into the endoplasmic reticulum re-
sulted in a restoration of processing function in BL cells by modulating the
peptide translocation into the MHC class I—containing compartment using
a TAP-independent pathway (Khanna et al., 1994). Furthermore, these BL
cells were successfully used as stimulators to generate virus-specific CTL
response in vitro. It is interesting that these effects were seen in the face of
down-regulated expression of adhesion molecules. The recent activation of a
tumor-specific response in vivo (Restifo et al., 1995) using this principle
gives some confidence that this approach may have some application to
EBV-associated tumors.

Another interesting approach to restore endogenous processing function is
to transfect gene(s) encoding EBV latent antigens into group I BL cells. This
approach is supported by the observation that activation of latent gene
expression in EBV-positive BL cells significantly increases susceptibility of
tumor cells to virus-specific cytolysis. Similarly, EBV-negative sporadic BL
cell lines, which often retain a group I phenotype on serial passage, can be
switched toward the group III phenotype by experimental infection with
EBV in vitro or by transfection with individual EBV latent genes (Rowe et
al., 1986; Torsteinsdottir et al., 1986; Wang et al., 1990). The most dramat-
ic single gene effects in this context are mediated by LMP1, which, if ex-
pressed in EBV-negative BL cells, induces a dose-dependent up-regulation of
adhesion molecules and B-cell activation antigens (Wang et al., 1990). Re-
cent studies have shown that transfection of the LMP1 gene in group I BL
cells consistently induces coordinate up-regulation of TAP-1, TAP-2, and
surface MHC expression. Furthermore, these effects were coincident with a
significant restoration of endogenous antigen processing as detected in CTL -
recognition assay (Rowe et al., 1995). Although the cell signaling pathways
whereby LMP1 mediates these diverse effects are only partly understood, it
is interesting to note that EBV LMP1 mimics the effects of interferon-γ in its
effects on TAP-1/2, HLA class I, HLA class II, ICAM1, and CD40 (Want et
al., 1990). These observations suggest some convergence between the LMP1
(Laherty et al., 1992; Hammarskjold and Simurda, 1992) and interferon-γ
(Loh et al., 1992) pathways. Clearly, the potential of EBV to modulate
antigen presentation in infected cells through one of its own gene products
has important implications for the immunogenicity of virus-positive malig-
nancies.

There is no convincing evidence that HD patients are unable to mount an
EBV-specific CTL response. It is thus not clear how RS cells, which are
known to express LMP, are able to escape recognition, since this protein is
known to include CTL epitopes (Khanna et al., 1992). The most likely
explanation is related to the lack of class I expression on RS cells, but the
presence of mutations in this protein might contribute as well. Perhaps the
most promising approach to immunotherapy of HD might be to utilize the

class II expression on RS cells. Of course, any strategy must take into account the substantial physical barrier presented by the CD4 cells of unknown function that surround the RS cells.

There are several other approaches in which it might be possible to harness the presence of EBV in these tumors. These strategies include genetic inactivation of EBNA1 (Roth et al., 1995) or activation of the EBV lytic cycle (Evans et al., 1995). For example, Fab fragments against CD15 (present on RS cells but not B cells) might be coupled to protamine and used to deliver a DNA plasmid (Chen et al., 1995) expressing BZLF1 (Prang et al., 1995).

2. EBV-INDEPENDENT THERAPEUTIC STRATEGIES

The apparent ability of BL to expand in the face of EBV-specific CTLs has focused attention on the use of LAK cells as a therapeutic tool. These LAK cells appear capable of killing BL cells in vitro (Misko et al., 1990). Given the recent modifications to natural killer cell–LAK-based strategies, which are designed to reduce toxicity, it seems justified to give serious consideration to treating BL patients in this manner, particularly in Papua New Guinea, where long-term survival is rare (Moss et al., submitted).

Other potential approaches include exploiting the presence of fetal, frameshifted, or overexpressed self-antigens (Lo et al., 1992; Townsend et al., 1994). Possible non-EBV targets include CD10 and CD77 (BL), Ki24 and Ki67 (NPC), and CD15 (HD). However, since some of these antigens are expressed on normal lymphoid tissue, raising an immune response to them may be difficult or hazardous. It is clearly not necessary to identify tumor antigens in order to develop a therapeutic strategy, since cognate antitumor immunity can be induced by transfection of tumor cells with cytokines and addition of co-stimulatory molecules (Bubenik, 1995). These approaches generally override the barrier for effective priming of an antitumor response.

NPC is derived from cells that are not professional antigen-presenting cells. The use of co-stimulatory molecules (e.g., B7) might thus offer the best opportunity for generating or priming a protective NPC-specific cellular response (Wu, 1994). The transduction of B7 gene could be achieved either by mixing the tumor cells with glycosylphosphatyl inositol (GPI)-anchored B7 (McHugh et al., 1995) or by directly injecting GPI-B7 into the tumor mass. The latter approach might be particularly suitable because NPC cells are difficult to culture.

As described earlier, deregulated cytokines and/or cell activation may be responsible for the HD lesion. Thus, approaches that neutralize or counteract critical cytokines or receptors (perhaps using targeted receptors or ligands) may be required, and appropriate genes might be delivered in vivo using DNA gun technology (Sun et al., 1995). Conceivably, the CD4 cells that surround the RS cells are tumor specific but are somehow anergized or

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have a Th2 phenotype. Thus, an approach might be to change the phenotype from Th2 (Trembleau et al., 1995) to Th1 using IL-12 (Tahara and Lotze, 1995)

C. Conclusion

This review has considered the issues of EBV vaccine development and immunotherapy in light of the different latency programs adopted in EBV-associated diseases. It has pursued the argument that the technology exists to begin human vaccine trials aimed at controlling IM and PTLD. Vaccine trials against other EBV-associated diseases may need to proceed with more caution and may be dependent on the emergence of novel vaccine strategies derived from either animal models or related viruses.

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Protective anti-tumor immunity induced by vaccination with recombinant adenoviruses encoding multiple tumor-associated cytotoxic T lymphocyte epitopes in a string-of-beads fashion

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ABSTRACT   Vaccines harboring genes that encode functional oncoproteins are intrinsically hazardous, as their application may lead to introduction of these genes into normal cells and thereby to tumorigenesis. On the other hand, oncoproteins are especially attractive targets for immunotherapy of cancer, as their expression is generally required for tumor growth, making the arisal of tumor variants lacking these antigens unlikely. Using murine tumor models, we investigated the efficacy of polyepitope recombinant adenovirus (rAd) vaccines, which encode only the immunogenic T cell epitopes derived from several oncogenes, for the induction of protective anti-tumor immunity. We chose to employ rAd, as these are safe vectors that do not induce the side effects associated with, for example, vaccinia virus vaccines. A single polyepitope rAd was shown to give rise to presentation of both H-2 and human leukocyte antigen-restricted cytotoxic T lymphocyte (CTL) epitopes. Moreover, vaccination with a rAd encoding H-2-restricted CTL epitopes, derived from human adenovirus type 5 early region 1 and human papilloma virus type 16-induced tumors, elicited strong tumor-reactive CTL and protected the vaccinated animals against an otherwise lethal challenge with either of these tumors. The protection induced was superior compared with that obtained by vaccination with irradiated tumor cells. Thus, vaccination with polyepitope rAd is a powerful approach for the induction of protective anti-tumor immunity that allows simultaneous immunization against multiple tumor-associated T cell epitopes, restricted by various major histocompatibility complex haplotypes.

Cytotoxic T lymphocyte (CTL)-mediated immunity plays an important role in the host defense against many tumors. However, natural CTL immunity against tumors often falls short in preventing the development of malignancies. Therefore, activation of the tumor-directed CTL response by vaccination constitutes a promising approach for the prevention and treatment of malignancies.

Induction of an effective CTL-mediated anti-tumor response against the relevant tumor-associated antigens can be achieved in various ways, including immunization with whole proteins, peptides. DNA encoding the tumor-associated antigen of choice, (genetically engineered) irradiated tumor cells, or attenuated organisms, like recombinant bacteria or viruses, expressing the tumor-associated antigens of choice (1–7). However, these vaccination approaches might not always result in the desired effect. For instance, vaccine approaches that deliver entire proteins may direct the immune response against

an immunodominant T cell epitope only and not against other subdominant T cell epitopes. Alternatively, such vaccines may direct the immune response primarily against epitopes that are subjected to antigenic variation (8–11). Induction of T cell immunity against preselected T cell epitopes through peptide vaccination has in certain cases been shown to induce protective anti-tumor immunity (2, 12), but it can also lead to specific CTL tolerance induction and, thereby, to enhanced tumor outgrowth (13, 14). In the latter instances, protective CTL-mediated immunity was induced when an adenovirus vector encoding the entire tumor antigen was used for vaccination.

Together, these observations prompted us to design a vaccine that would deliver the T cell epitopes via a mode ensuring the induction of protective immunity and that is rationally designed, in the sense that only minimal essential epitopes derived from one or more tumor-associated antigens expressed by the same tumor are incorporated. The approach of using the sequences encoding immunogenic T cell epitopes derived from (onco)proteins, rather than the entire proteins, has the additional advantage of avoiding the potential hazards of immunizing with agents carrying genes that are, or may be, involved in transformation. For example, recombinant vaccines for cervical carcinoma are intrinsically hazardous if such vaccines contain the functional human papilloma virus type 16 (HPV16) early region 6 (E6) and E7 oncogenes (15, 16). The same holds true for vaccines encoding other proteins that are implicated in the development of malignancies, such as HER2/neu, the aberrant fusion protein BCR-ABL, or mutated Ras and p53 proteins. Likewise, the use of vectors encoding tumor-specific antigens of which the function has not yet been elucidated, such as the MAGE genes, has a potential risk, as the consequences of (over)expression of such genes are unknown. Through application of recombinant vectors encoding string-of-beads arrangements of oncogene-derived T cell epitopes, as opposed to vectors harboring intact oncogenes, the introduction of functional oncogenes into the patient is avoided.

Recently, recombinant vaccinia viruses expressing several virus-derived CTL epitopes in a string-of-beads fashion have been successfully used to immunize mice (17, 18). These studies show the potency of the use of string-of-beads vaccines for the induction of antiviral immunity. However, the potential risks associated with vaccinia virus, such as encephalopathy and postvaccinal encephalitis, as well as the decreasing or absent (in younger individuals) immunity to poxvirus, prohibit the large scale application of recombinant vaccinia vaccines in

Abbreviations: Ad5E1A, adenovirus type 5 early region 1A; B6, C57BL/6; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; HLA, human leukocyte antigen; HPV 16, human papilloma virus type 16; MEC, mouse embryo cells; MHC, major histocompatibility complex; moi, multiplicity of infection; rAd, recombinant adenovirus(es); TNF, tumor necrosis factor.
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humans (19). High postvaccination complication rates are reported, particularly in older (those aged >4 years) primary vaccinees (20). Therefore, we studied the potential of a recombinant adenovirus vector (rAd) encoding several CTL epitopes in a string-of-beads fashion. Attractive features of adenovirus-based vaccines are their well-characterized genetic arrangement and function, as well as their extensive and safe usage in North American army recruits without inducing adverse side effects (21–23). The safety of the currently used rAd is even increased by the fact that these vectors, due to deletion of the E1 region, are largely replication defective.

Recently, promising results have been obtained by us and others using rAd encoding intact tumor-associated antigens as vaccines for the induction of protective anti-tumor immunity (13, 14, 24, 25). We now demonstrate that a rationally designed rAd vaccine, encoding a string-of-beads arrangement of several CTL epitopes derived from the human adenovirus type 5 early region 1A (Ad5E1A), E1B and HPV16 E7 oncoproteins, is a highly effective tool for the induction of protective T cell-mediated anti-tumor immunity.

MATERIALS AND METHODS

Cell Lines. All cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) (Seromed, Berlin) supplemented with 4% fetal calf serum (HyClone), penicillin (110 international units/ml; Brocades Pharma, Leiderdorp, The Netherlands), and 2-mercaptoethanol (20 μM) at 37°C in a 5% CO_2 atmosphere. CTL clones were cultured as described elsewhere (26–29).

Generation of rAd. The adenoviral vector construction adapter plasmid pMad5 was derived from plasmid pMLP10 (30) as follows. pMLP10-lin was constructed by insertion of a synthetic DNA fragment with unique sites for the restriction endonucleases *Mlu*I, *Spl*I, *Sna*BI, *Spe*I, *Asu*II, and *Mun*I into the *Hin*dIII site of pMLP10. Subsequently, the adenovirus *Bgl*II fragment spanning nucleotides 3328–8914 of the Ad5 genome was inserted into the *Mun*I site of pMLP-lin. Finally, the *Sal*I–*Bam*HI fragment was deleted to inactivate the tetracycline resistance gene, resulting in plasmid pMad5. A minigene cassette vector, pMad5-0, was generated by ligation of the annealed and phosphorylated double-stranded oligonucleotides 1a/b and 2a/b (see Table 1) into the *Mlu*I and *Spe*I sites of pMad5. This cloning step leads to elimination of the original *Mlu*I and *Spe*I sites and to creation of a small ORF, which essentially consists of a start codon, the sequence SEQKLISEEDLNN, a human c-Myc-derived sequence, which is recognized by mAb 9E10 (31) and a stop codon. A small "stuffer"
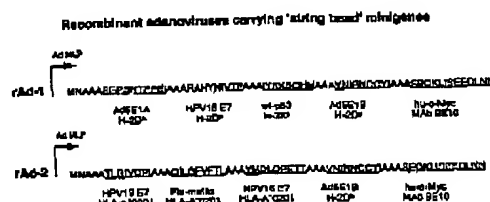
FIG. 1. Minigenes encoding several CTL epitopes, linked by a spacer of three alanines. The first minigene (rAd-1) encodes the H-2D^b-restricted peptide E1A$^{234-243}$ (26), the H-2D^b-restricted peptide HPV16E7$^{49-57}$ (2), the H-2K^b-restricted peptide p53$^{158-166}$ (49), the H-2D^b-restricted peptide E1B$^{192-200}$ (27), and a Myc tag (31). The second minigene (rAd-2) encodes the HLA-A*0201-restricted CTL epitope HPV16 E7$^{86-93}$ (37), the HLA-A*0201-restricted peptide Flu-matrix$^{58-66}$ (48), the HLA-A*0201-restricted peptide HPV16 E7$^{11-30}$ (37), the H-2D^b-restricted peptide E1B$^{192-200}$ (27), and a Myc tag (31). Both minigenes are place behind the Ad5 major late promoter (Ad-LMP), which in this configuration is linked to the Ad5 immediate early enhancer, resulting in immediate early expression of the minigenes.

sequence, flanked by newly generated *Mlu*I and *Spe*I sites, is present between the start codon and the c-Myc sequence.

pMad5-1 and -2, each of which harbor a multi-epitope-encoding minigene, were constructed by unidirectional cloning of the following double-stranded oligonucleotides into pMad5-0, which had been cleaved with *Mlu*I and *Spe*I. pMad5-1: numbers 5a/b, 6a/b, 7a/b, 4a/b; pMad5-2: numbers 3a/b, 8a/b, 9a/b, 4a/b (see Table 1). After each cloning step, the sequence of the inserts was verified by DNA sequencing. The recombinant proteins that are encoded by the resulting minigenes are depicted in Fig. 1. Expression of these minigenes is driven by the Ad5 major late promoter, which in this configuration is linked to the Ad5 immediate early enhancer, resulting in immediate early expression of the minigenes.

rAds were generated through *in vivo* homologous recombination in the Ad5E1-transformed helper cell line 911 (29) between plasmid pJM17 (32), containing the sequence of the Ad5 mutant dl309, and either of the plasmids pMad5-1 or pMad5-2. 911 cells were transfected with 10 μg of plasmid pJM17 in combination with 10 μg of either pMad5-1 or pMad5-2. The rAds were plaque-purified three times, after which the clonal rAds were propagated in 911 cells, purified by double cesium chloride density gradient centrifugation, and extensively dialyzed. The presence of replication-competent adenoviruses was routinely checked by infection of Hep-G2 cells. The viral stocks were stored in aliquots with 10% glycerol

Table 1. Oligonucleotides used for the generation of rAdV

1a	CGCGAATTATGAACGCGTC
1b	GTACGACGCGTTCATAATT
2a	GTACGCTACTAGTGAACAGAAGCTGATATCAGAGGAAGACCTAAACTGAT
2b	CTAGATCAGTTTAGGTCTTCCTCTGATATCAGCTTCTGTTCACTAGTAGC
3a	CGCGGCAGCTTCCGGTCCTTCTAACACACCTCCTGAGATAGCAGCC
3b	GCTATCTCAGGAGGTGTGTTAGAAGGACCGGAAGCTGC
4a	CTGTAAATATCAGGAATTGTTGCTACATTGCAGCTG
4b	CTAGCAGCTGCAATGTAGCAACAATTCCTGATATTTACAGCTGC
5a	CGCGGCAGCTACACTAGGAATTGTGTGCCCCATCGCAGCC
5b	GCGATGGGGCACACAATTCCTAGTGTAGCTGC
6a	GCTAGAGCCCATTACAATATTGTAACCTTTGCTGCG
6b	GCAAAGGTTACAATATTGTAATGGGCTCTAGCGGCT
7a	GCTGCCATCTACAAGAAGTCACAGCACATGGCTGCAG
7b	ACGCCGACGGTAGATGTTCTTCAGTGTCGTGTACCGA
8a	GCTGGAATCCTAGGTTTCGTCTTTACGCTAGCTGCG
8b	GCTAGAGTAAAGACGAAACCTAGGATTCCAGCGGCT
9a	GCTTATATGTTAGATTTGCAACCAGAGACAACTGCTGCAG
9b	AGCAGTTGTCTCTGGTTGCAAATCTAACATATAAGCCGCA

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at −80°C and titered by plaque assay using 911 cells as described in ref. 29.

Transfection of COS-7 Cells. Transient transfection in COS-7 cells was performed as described elsewhere (33). In short, 100 ng of plasmids encoding Ad5E1, HPV16 E7, murine p53, or the influenza-matrix protein together with 100 ng of a plasmid encoding H-2D^b, H-2K^b, or HLA-A*0201 was transfected into 1 × 10^6 COS-7 cells. The transfected COS cells were incubated in 100 μl of IMDM containing 8% fetal calf serum for 48 h at 37°C, after which 1500–5000 CTL in 25 μl of IMDM containing 50 Cetus units (= 300 international units) of recombinant interleukin-2 (Cetus) were added. After 24 h, the supernatant was collected, and its tumor necrosis factor (TNF) content was determined by measuring its cytotoxic effect on WEHI-164 clone 13 cells as previously described (33).

Infection of Mouse Embryo Cells (MEC) with rAd. C57BL/6 (B6) MEC or HLA-A2-positive SAOS cells (human osteosarcoma cells) were infected with rAd diluted in 1 ml of IMDM containing 0.5% BSA. After 30 min at room temperature, 1 ml of IMDM containing 10% fetal calf serum was added. The multiplicity of infection (moi; B6 MEC, 50; SAOS, 10) was chosen to give at least 80% infected cells. The desired moi was determined by infecting these cells with Ad.RSVβ-Gal, a rAd carrying the *Escherichia coli lacZ* gene under control of the promotor from the Rous sarcoma virus long terminal repeat, followed by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining 48 hr later.

Generation of CTL Bulk Cultures. Spleen cells, 5 × 10^6 per well, derived from B6 mice taken 2 weeks or more after intraperitoneal immunization with 1 × 10^8 plaque-forming units of rAd or the replication-defective adenovirus type 5 mutant Ad5ts 149, were cocultured for 6 days with 10% irradiated (25 Gy) interferon-γ (10 units/ml)-treated stimulator cells in 24-well plates. Effector cells were used in a cell-mediated lymphocyte cytotoxicity assay as previously described (27).

Peptides. Peptides were generated by solid phase strategies on a multiple peptide synthesizer (Abimed AMS 422; Langenfeld, Germany) as described previously (34).

Tumor Cell Challenge. B6 mice were immunized intraperitoneally with 1 × 10^8 plaque-forming units of rAd or the Ad5 mutant Ad5ts149 in 0.25 ml of PBS/BSA. Two weeks later, the mice were subcutaneously challenged with 0.4 × 10^6 Ad5E1A + Ras cells or 0.5 × 10^6 HPVC3 cells in 0.25 ml of PBS. Tumor volumes were measured with a caliper. Animals were sacrificed when their tumors grew larger than 1,000 mm³ to avoid unnecessary suffering.

RESULTS

The CTL Epitopes Encoded by the Constructed rAd Are Processed and Presented to Tumor-Specific CTL. Vaccination with recombinant viruses encoding functional oncoproteins is intrinsically hazardous because it can lead to introduction of oncogenes into somatic cells and, thereby, to the development of tumor cells at the site of injection. To circumvent this problem, we constructed rAd that carried synthetic minigenes encoding a string-of-beads arrangement of minimal CTL epitopes derived from several of such oncogenes. First, minigenes were assembled in shuttle vector pMad5. Minigene 1 was designed to encode four H-2-restricted CTL epitopes that were presented by different murine tumors of B6 origin (Fig. 1), whereas minigene 2 was designed to encode the H-2D^b-restricted E1B$^{192-200}$ peptide (as positive control) and three HLA-A*0201-binding peptides (Fig. 1). A crucial aspect of the design of these string-of-beads minigenes is to permit proper processing of the recombinant proteins into the different immunogenic peptides. As antigenic sequences flanked by multiple alanines were shown previously to become efficiently

processed relatively independent of the protein context (35), we chose to separate the CTL epitopes from each other by a spacer of three alanines.

To test whether the constructed minigenes are able to generate the CTL epitopes concerned, they were transfected into COS-7 cells together with plasmids encoding the relevant major histocompatibility complex (MHC) class I restriction elements. The transfected COS-7 cells were used in a TNF production assay as stimulator cells for different CTL clones recognizing the epitopes present in the minigenes. These experiments showed that all four epitopes encoded by minigene 1, and at least two of the four CTL epitopes, for which specific CTL clones were available (E1B$^{192-200}$ and Flu$^{58-66}$), encoded by minigene 2 were properly processed and presented to tumor-specific CTL (data not shown).

Subsequently, the minigenes were used to generate replication-defective B6 MEC or HLA-A*0201-positive SAOS cells infected with these rAd were used as stimulator cells in a TNF production assay to analyze whether the rAd-infected cells were able to specifically activate the tumor- and virus-reactive CTL clones. Upon infection with rAd carrying minigene 1 (rAd-1), peptides E1A$^{234-243}$, E1B$^{192-200}$, and HPV16 E7$^{49-57}$ were presented by the infected cells, as the corresponding CTL were activated when incubated with B6 MEC infected with this virus but not when incubated with B6 MEC infected with a control rAd (Fig. 2). By infection of MEC derived from p53 knockout mice, we were able to show that also peptide p53$^{158-166}$ was efficiently processed and presented to p53-specific CTL (data not shown). Likewise, the rAd encoding minigene 2 (rAd-2) was able to deliver peptides E1B$^{192-200}$ and Flu$^{58-66}$, to the surface of the infected cells in the context of H-2D^b (Fig. 2) and HLA-A*0201 (Fig. 3), respectively. Processing and presentation of the HPV16 E7-derived HLA-A*0201-restricted CTL epitopes incorporated in rAd-2 could not be tested, because no CTL clones specific for these peptides are currently available. In conclusion, rAd-1 and rAd-2 are capable of delivering all preselected H-2b-restricted CTL epitopes to tumor-specific CTL, as well as the HLA-A*0201-restricted peptide Flu$^{58-66}$.

Vaccination of B6 Mice with rAd Induces Tumor-Reactive CTL Activity. Because we found rAd-1 and rAd-2 to deliver all H-2b-restricted CTL epitopes, we analyzed whether vaccination with these viruses would induce CTL activity against the epitopes concerned. Indeed, bulk T cell cultures derived from B6 mice immunized with rAd-1 showed high CTL activity
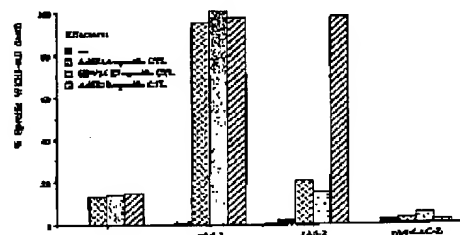
FIG. 2.   CTL epitopes encoded by rAd were processed and presented to tumor-specific CTL. B6 MEC were left uninfected or were infected with rAd-1 harboring minigene 1, rAd-2, harboring minigene 2, or the galactosidase gene (rAdV-LAC-Z). After 48 hr, the infected MEC were tested for the expression of the CTL epitopes in their ability to cause TNF release by the relevant CTL. The presence of TNF in the culture supernatant was measured by the cytotoxic effect on WEHI-164 clone 13 cells. B6 MEC, infected with rAd-1 harboring E1A$^{234-243}$, HPV16 E7$^{49-57}$, and E1B$^{192-200}$-derived H-2D^b-restricted CTL epitopes, are able to activate CTL clones specific for these CTL epitopes whereas B6 MEC infected with rAd-2 harboring the E1B$^{192-200}$ epitope only activate Ad5E1B-specific CTL. The CTL are not activated upon incubation with uninfected MEC or MEC infected with a control rAd.
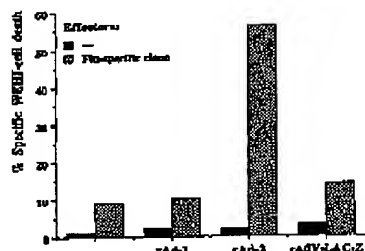
FIG. 3. The Flu-derived epitope encoded by rAd-2 is processed and presented to Flu-specific CTL. HLA-A*0201-positive SAOS cells were infected with rAd and used as stimulator cells in a TNF release assay. SAOS cells infected with rAd-2, but not with rAd-1 or rAd-LAC-Z, activate the Flu-specific CTL clone Q66.9, indicating that the Flu-derived CTL epitope is processed and presented.

against target cells loaded with the peptides E1A$^{234-243}$, E1B$^{192-200}$, and HPV16 E7$^{49-57}$ (Figs. 4 and 5). Importantly, the resulting CTL also displayed strong anti-tumor reactivity, in that tumor cells presenting the relevant CTL epitopes as endogenously processed antigen were efficiently lysed. We were not able to show p53-specific CTL reactivity under the experimental conditions used (data not shown), possibly as a result of tolerance to this self-peptide. Vaccination of B6 mice with rAd-2 induced CTL activity only against E1B$^{192-200}$ and not against the E1A$^{234-243}$ or HPV16 E7$^{49-57}$ epitopes (Fig. 5), demonstrating that immunization with rAd specifically induces CTL reactivity against the epitopes encoded by the respective minigenes. From these data, we conclude that vaccination with polyepitope rAd, which encode a recombinant polypeptide comprising several CTL epitopes in a string-of-beads fashion, induces strong tumor-specific CTL responses against the preselected epitopes.

Induction of Protective Anti-Tumor Immunity by Vaccination with rAd. As immunization of B6 mice with rAd-1 induces both E1A- and HPV16 E7-specific CTL-mediated immunity, we studied whether this rAd-induced immunity would protect mice against a lethal challenge with tumor cells expressing the
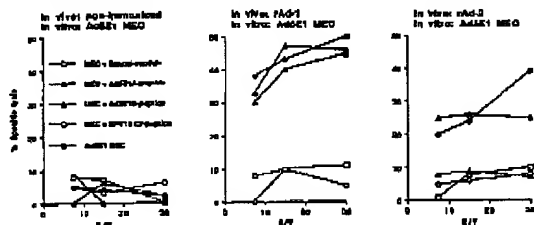
FIG. 4. Vaccination with rAd leads to induction of tumor-reactive CTL activity against the E1-derived CTL epitopes. B6 mice were left nonimmunized, were immunized with rAd-1, harboring minigene 1, or were immunized with rAd-2, harboring minigene 2. Two weeks later, spleen cells of these animals were taken and restimulated with Ad5E1-transformed tumor cells to propagate E1A- and E1B-specific CTL. Lytic activity of bulk CTL cultures was tested 6 days later on Ad5E1 MEC, untransformed B6 MEC loaded with the Sendai-virus encoded control CTL epitope FAPGNYPAL, the E1A-encoded CTL epitope, the E1B-encoded CTL epitope, or the HPV16 E7-encoded CTL epitope. Mice immunized with rAd-1 recognize the E1A- and E1B-encoded CTL epitopes as well as tumor cells endogenously presenting these epitopes. Mice immunized with rAd-2 recognize the E1B epitope as well as tumor cells endogenously presenting this CTL epitope, whereas nonimmunized mice do not display reactivity against the target cells. Percent specific lysis at different effector to target cell ratios is shown.
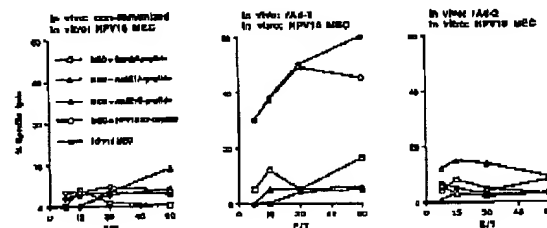
FIG. 5. Vaccination with rAd leads to the induction of tumor-reactive CTL activity directed against the HPV16 E7, H-2D^b-restricted CTL epitope. B6 mice were left nonimmunized, were immunized with rAd-1, harboring minigene 1, or were immunized with rAd-2, harboring minigene 2. Two weeks later, spleen cells of these animals were taken and restimulated with HPV16-transformed tumor cells to propagate H-2D^b-restricted, HPV16 E7-specific CTL. Lytic activity of bulk CTL cultures was tested 6 days later on HPV16 MEC, untransformed B6 MEC loaded with the Sendai-virus encoded control CTL epitope FAPGNYPAL, the E1A-encoded CTL epitope, the E1B-encoded CTL epitope, or the HPV16 E7-encoded CTL epitope. Mice immunized with rAd-1 recognize the HPV16 E7-encoded CTL epitope as well as tumor cells endogenously presenting the HPV16 E7 epitope. Nonimmunized mice and mice immunized with rAd-2 do not display reactivity against HPV16 E7 peptide positive target cells. Percent specific lysis at different effector to target cell ratios is shown.

relevant oncogenes. rAd-immunized mice were challenged with either Ad5E1A + EJras- (13) or HPV16 + EJras-transformed (2) tumor cells. Indeed, mice immunized with rAd-1, in contrast to mice immunized with rAd-2 (not harboring the E1A epitope) or PBS/BSA, were protected against the outgrowth of Ad5E1A + Ras tumor cells (Fig. 6). Moreover, the protection induced by vaccination with rAd-1 was more pronounced than that obtained by vaccination with irradiated tumor cells, showing that vaccination with rAd is superior compared with vaccination with irradiated tumor cells. Mice immunized subcutaneously or intramuscularly are as equally well protected as mice immunized intraperitoneally (Fig. 7), demonstrating that rAd function as effective antitumor vaccines when administered via different routes.

rAd-1-immunized mice were protected not only against Ad5E1A + Ras tumors but also against an otherwise lethal challenge with HPV16 + Ras-transformed tumor cells (Table 2). Thus, vaccination with polyepitope rAd constitutes a powerful method to induce protective anti-tumor immunity
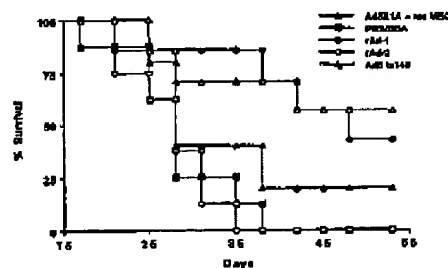
FIG. 6. Vaccination with rAd-1 induces protective immunity against a lethal challenge with Ad5E1A-expressing tumor cells. B6 mice were immunized intraperitoneally with rAd-1 ($n = 7$), rAd-2 ($n = 8$), or the Ad5 mutant (Ad5E1A-positive) Ad5sr149 ($n = 7$) or were injected with PBS/BSA ($n = 8$) only. Two weeks later, the mice received a subcutaneous challenge of 0.4×10^6 Ad5E1A + Ras cells. Mice immunized with rAd-1 and Ad5sr149 were protected against the outgrowth of Ad5E1A + EJras cells, showing that immunization with rAd induces protective immunity against tumors.

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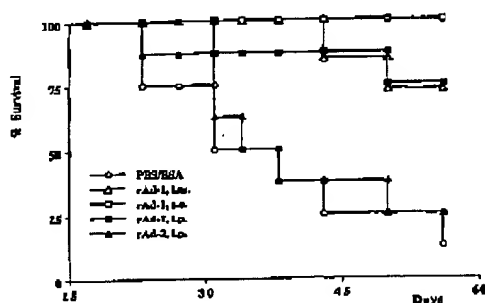
FIG. 7.   Different routes of vaccination with rAd induce protective anti-tumor immunity. B6 mice were immunized intraperitoneally (i.p.) (*n* = 8), intramuscularly (i.m.) (*n* = 8), or subcutaneously (s.c.) (*n* = 8) with rAd-1, immunized with PBS/BSA (*n* = 8), or immunized i.p. with rAd-2 (*n* = 8). Two weeks later, the mice received a subcutaneous challenge of 0.4 × 10⁶ AdSE1A + Ras cells. Mice immunized intraperitoneally, intramuscularly, or subcutaneously with rAd-1 are protected against AdSE1A + E1ras cells.

directed against multiple selected CTL epitopes derived from different oncogene products.

DISCUSSION

A single vaccination with rAd encoding multiple CTL epitopes, derived from different oncoproteins and arranged in a string-of-beads fashion, can elicit CTL responses against these epitopes. The resulting CTL are capable of lysing tumor cells presenting these peptides. The ability to induce CTL responses against oncoprotein-derived peptides by immunization with such polyepitope rAd offers a clear advantage over immunization strategies in which vectors carrying functional oncogenes are used, in that it largely eliminates the risk of transformation of recombinant vector-infected cells. Moreover, CTL activity can be induced against multiple (onco)gene products expressed by the same tumor cell, as illustrated by the induction of both E1A- and E1B-specific immunity after immunization with rAd-1. The similar approach can be taken for cervical carcinoma. In the majority of cervical carcinomas, both the HPV-encoded oncoproteins E6 and E7 are constitutively expressed because they are required for maintenance of the transformed state (15, 16). By introducing into rAd the HPV16 E6 and E7-derived CTL epitopes that bind to HLA-A1, A2, A3, A11, and A24 (36, 37), a powerful and versatile polyepitope vaccine against HPV16-induced cervical carcinoma may be created. As this vaccine would induce CTL

Table 2. Vaccination with rAd-1 induces protective immunity against HPV16-transformed tumors

Treatment	Tumor-take*
Exp. 1	
None	8/10
rAd-1	2/10
Ad5ts149	6/10
Exp. 2	
None	10/11
rAd-1	3/10
Ad5ts149	10/10

B6 mice were left untreated or were immunized intraperitoneally with 1 × 10⁸ plaque-forming units of rAd-1 or Ad5ts 149 as control. Two weeks later, the mice received a subcutaneous challenge of 0.5 × 10⁶ HPV16-transformed tumor cells (2). (8/10 vs. 2/10, *P* < 0.05; 10/11 vs. 3/10, *P* < 0.01; Tukey–Kramer multiple comparisons test.)
*Tumor-take is depicted at 3 weeks after tumor cell challenge as tumor-bearing animals/number of mice.

immunity against multiple epitopes derived from two proteins required by the tumor for malignant growth, the risk of tumor-immune escape by antigen loss or antigen mutation is relatively small. Furthermore, based on the occurrence of the different HLA-A alleles in the caucasian population, this vaccine could be applied to the majority (>90%) of people in this population.

Many of the CTL epitope-specific vaccines directed against oncogenic proteins, which have been tested in previous studies, were based on synthetic peptides. Although in many cases peptide-based anti-tumor vaccination strategies were successful (2, 12), in other cases such vaccines were shown to induce CTL unresponsiveness, leading to enhanced tumor outgrowth (13, 14). The latter was shown by immunization with synthetic peptides representing the CTL epitopes E1A²³⁴⁻²⁴³ or E1B¹⁹²⁻²⁰⁰, the same peptides that are incorporated in our rAd vaccines. Vaccination with these peptides induced a functional deletion of E1A- and E1B-specific CTL, respectively, and led to enhanced outgrowth of Ad5E1(A)-transformed tumor cells. We now show that these CTL epitopes, when delivered by rAd, induce protective anti-tumor immunity, demonstrating the advantage of rAd over synthetic peptides for use in epitope-specific vaccination.

Two of the four CTL epitopes present in rAd-1, E1A²³⁴⁻²⁴³ and HPV16 E7⁴⁹⁻⁵⁷, induce only suboptimal CTL responses when irradiated tumor cell are used for vaccination (5, 14, 28). The CTL response induced by immunization with HPV16-induced tumor cells is predominantly directed against a hitherto unidentified CTL epitope (28), whereas immunization with Ad5E1-transformed tumor cells, expressing both the E1A- and E1B-encoded CTL epitopes, is primarily directed against peptide E1B¹⁹²⁻²⁰⁰ (5, 14). The "subdominant" nature of the E1A epitope, when present in a tumor cell vaccine, is also reflected by the fact that vaccination with irradiated Ad5E1A-expressing tumor cells induces only a marginal protection against a challenge with Ad5E1A + Ras cells. In contrast, vaccination with rAd-1 elicits strong "co-dominant" CTL responses directed against all three CTL epitopes.

For optimal CTL induction by vaccines that encode several CTL epitopes in a string-of-beads fashion, it is obligatory that all CTL epitopes are properly processed and presented in the context of MHC class I glycoproteins. In the MHC class I pathway of antigen presentation, cellular proteins are degraded in the cytosol, resulting in peptides that are transported by TAP into the endoplasmatic reticulum (ER). Here, they assemble with the MHC class I heavy chain and β₂-microglobulin to the trimolecular MHC complex (38). Consequently, the efficiency by which proteolytic degradation in the cytosol and transportation of the resulting peptides into the ER takes place is a key factor in determining whether a given peptide is presented in the context of MHC class I molecules. Indeed, B6 MEC derived from transporter associated with antigen processing (TAP)−/− mice, when infected with rAd-1 or rAd-2, were not able to stimulate the E1A-, HPV16 E7-, p53-, or E1B-specific CTL (data not shown), indicating that TAP-mediated transport of the rAd-encoded CTL epitopes into the ER is mandatory. One of the major proteolytic systems responsible for degradation of cytosolic proteins is the proteasome complex (39). It has been shown that the cleavage preference of the proteasome can define the antigenic potential of a protein (40) or even result in a lack of antigen presentation of CTL epitopes (41). Our data indicate that in polyepitope rAd-infected cells, the generation and subsequent transportation of the CTL epitopes was efficient, ensuring CTL activation *in vitro* and *in vivo*. Nonetheless, flanking the CTL epitopes with proteasomal cleavage sequences other than the triple alanine used in our minigene constructs might further improve the ability of polyepitope rAd to elicit immune responses (42).

The ability to produce large quantities of purified virus with relative ease makes rAd very attractive for clinical use. Most importantly, rAd can be safely applied to human beings because they rarely integrate into the host genome and therefore have little chance to activate a dormant oncogene or disable a tumor suppressor gene (43). Adenoviruses are extensively and safely used in vaccination of North American army recruits without causing side effects (21, 22). Furthermore, the use of rAd vectors from which the E1 region is deleted minimizes the risk of propagation of the virus, as replication of such viruses under physiological conditions is severely compromised. Recently, rAd have been applied both in nonhuman primates and in phase I clinical trials for the correction of the genetic deficiency underlying cystic fibrosis without inducing any severe side effects or toxicity (44–47). Using well defined murine tumor models, we now demonstrate that polyepitope rAd constitute powerful vaccines that can be employed for the induction of strong tumor-protective CTL-mediated immunity directed against different transforming oncogene products. Tumor protection was not only induced by intraperitoneal injection of rAd but also after vaccination routes that are easy to carry out in patients (subcutaneous and intramuscular). Polyepitope rAd, therefore, have significant potential for use as vaccines in the immunotherapy of cancer, as well as in infectious diseases such as AIDS, which requires multiple T cell responses to be activated and boosted.

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HUMAN CYTOTOXIC T LYMPHOCYTE RESPONSES TO EPSTEIN-BARR VIRUS INFECTION

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ABSTRACT

Epstein-Barr virus (EBV) provides one of the most informative systems with which to study cytotoxic T lymphocyte (CTL) responses in humans. The virus establishes a highly immunogenic growth-transforming infection of B lymphocytes, associated with the coordinate expression of six virus-coded nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, -LP) and two latent membrane proteins (LMPs 1 and 2). This elicits both primary and memory CT8$^+$ CTL responses that are markedly skewed toward HLA allele-specific epitopes drawn from the EBNA3A, 3B, 3C subset of latent proteins, with reactivities to other antigens being generally much less frequent. This heirarchy of immunodominance among the different latent proteins may at least partly reflect their differential accessibility to the HLA class I–processing pathway. Furthermore, CTLs to some of the immunodominant epitopes involve highly conserved T cell receptor (TCR) usage, a level of focusing which evidence suggests could have immunopathological consequences from cross-reactive recognition of other target structures. EBV is associated with a range of human tumors, and there is increasing interest in the possibility of targeting such malignancies using virus-specific CTLs. The dramatic reversal of EBV-driven lymphoproliferations in bone marrow transplant patients following CTL infusion demonstrates the potential of this approach, and here we discuss prospects for its extension to other EBV-positive tumors in which the immunodominant EBNA3A, 3B, 3C proteins are not expressed.

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INTRODUCTION

Epstein-Barr virus (EBV), a human gamma herpesvirus with a marked tropism for B lymphocytes, has potent cell growth–transforming ability yet is carried by the vast majority of individuals as a life-long asymptomatic infection. The growth-transforming function of the virus maps to a specific subset of genes, the latent genes, which are coordinately expressed in the latently infected lymphoblastoid cell lines (LCLs) that arise following viral infection of resting B cells in vitro. These genes encode eight latent proteins, the nuclear antigens EBNAs 1, 2, 3A, 3B, 3C, and -LP (sometimes referred to as EBNAs 1, 2, 3, 4, 6, and 5, respectively) and the latent membrane proteins LMPs 1 and 2. Allelic polymorphisms in the EBNA2, 3A, 3B, and 3C genes define two broad types of EBV isolate, types 1 and 2 (sometimes referred to as A and B), which show subtle differences in transforming function. The role of the latent proteins in the establishment/maintenance of the transformed state has recently been reviewed (1), and here we concentrate only on their importance as targets for immune recognition.

Work on the immunology of EBV infection has for many years gone hand in hand with virological studies examining the biology of the virus-host interaction. This broader scenario is also the subject of a recent review (2) from which the following essential points can be drawn. In most human populations, primary EBV infection occurs during the first few years of life, when it is almost always asymptomatic. The tendency toward delayed primary infection now being seen in Western societies, therefore, constitutes a novel situation in evolutionary terms and, very interestingly, a proportion of these late infections are clinically manifest as infectious mononucleosis (IM). During primary infection, orally transmitted virus replicates locally in oropharyngeal epithelium, with expression of the full array of replicative (lytic cycle) genes (3), and the virus also colonizes the lymphoid system through virus-driven expansion of infected B cell clones selectively expressing the latent cycle genes (4). This results in a life-long virus carrier state in which latently infected B cells constitute the reservoir upon which viral persistence depends; periodic reactivation of B cells from latency into lytic cycle can then reinitiate replicative foci, leading to low-level shedding of infectious virus from mucosal surfaces throughout life (2).

Host immune responses are thought to be of central importance both in limiting the primary infection and in controlling the lifelong virus carrier state. Serological studies have shown that the primary and persistent phases of infection are associated with different combinations of antibody reactivities to lytic and latent antigens (2). Of these, antibodies to the major virus envelope glycoprotein gp340 warrant particular attention because of their capacity both to neutralize viral infectivity (5, 6) and to mediate antibody-dependent cellular

cytotoxicity against cells in the late phase of virus replication (7). The balance of evidence suggests that such responses do not play a major role in controlling an already established EBV infection, although they may be important in rendering such an individual immune to subsequent infection with additional exogenously transmitted virus strains. Even here, however, it is questionable whether humoral immunity alone is sufficient to confer protection (8). By contrast, the frequency with which EBV-positive lymphoproliferative disease (9) and clinically apparent virus replicative lesions (10) are seen in T cell–immunocompromised patients strongly suggests an important role for cell-mediated immune responses in the control of EBV. A number of effector mechanisms are likely to be involved in this context, and some appreciation of their diversity comes from the variety of reports in which other cell types within blood mononuclear cell preparations can either delay or prevent the virus-induced in vitro transformation of resting B cells (2). Several of these effects are seen with nonimmune as well as with virus-immune donors, and they appear to be mediated by cytokine release from nonspecifically activated T or NK-like cells. The most obvious disturbance of in vitro transformation, however, is the regression of B cell outgrowth mediated by virus-specific cytotoxic T lymphocytes (CTLs), a phenomenon seen only in cultures from virus-immune individuals and one that provides the first evidence of long-term CTL surveillance over EBV infection (11). It is this type of immunity that has received most attention in recent years and that is the subject of this review.

MEMORY CTL RESPONSES

Antigen Choice

Memory CTLs in the blood of healthy virus carriers can be reactivated in vitro by stimulation with the autologous LCL and expanded as polyclonal T cell lines or as CTL clones in interleukin 2 (IL2)–conditioned medium (12–14). The bulk of the effectors thus produced are CD8$^+$ T cells that recognize the autologous LCL but not autologous mitogen-activated B lymphoblasts and that display HLA class I restriction in their pattern of reactivity against allogeneic LCLs. The response also contains a much smaller CD4$^+$ HLA class II–restricted cytotoxic component, apparent under certain conditions of in vitro reactivation (15), but these effectors are much less well characterized. When the antigenic specificity of the dominant CD8$^+$ CTLs was assayed on targets expressing individual EBV latent proteins from recombinant vaccinia vectors (16), even the earliest studies showed a marked skewing of responses toward the EBNA3A, 3B, 3C subset of antigens (17–19). Since then the analysis has been extended to more than 60 healthy virus carriers representing a range of different HLA

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types, and in the great majority of cases, the dominant CTL reactivities are again directed toward one or more of the EBNA3A, 3B, or 3C proteins. In some cases, subdominant responses recognizing other latent proteins have been detected; the most frequent target is LMP2, less frequently EBNA2, EBNA-LP, or LMP1, and very rarely if ever EBNA1 (17–20, and references below).

These apparent differences in the immunogenicity of EBV latent proteins are interesting, but it should be stressed that studies to date have concentrated largely on Caucasian donors and on CTL responses to LCLs transformed with type 1 virus, the prevalent virus type in Western societies. Furthermore it is possible that the overall pattern of results may be disproportionately influenced by certain HLA alleles such as A3, A11, B7, B8, and B44, which are relatively common in Caucasian populations, which tend to act as strong restriction elements in the EBV system, and which present target antigens from the EBNA3A, 3B, 3C subset (18, 21–26). Interestingly, another common HLA allele, A.1, has never been observed to mediate a response in this viral system, while the most frequent allele, A2.01, tends to be a weak restriction element inducing minor reactivities against epitopes from EBNA3C or LMP2 (27–29). Recent evidence in fact suggests that HLA-B alleles may generally be more efficient than HLA-A alleles in their loading and transport of viral peptides (R Khanna, DJ Moss, unpublished data).

Antigen Processing

The concept of a hierarchy of immunodominance among the EBV latent antigens is therefore a provisional one and needs to be tested further in other human populations with a different range of HLA alleles and a different spectrum of resident virus strains. Nevertheless, one of the most striking findings to emerge from the above work, namely the extreme rarity of CD8$^+$ CTL responses to EBNA1, has been further pursued in a recent study which suggests that an internal glycine-alanine repeat (GAr) domain protects the EBNA1 protein from the HLA–class I pathway of antigen presentation (30). Thus, inserting a known CTL epitope into the EBNA1 primary sequence renders it not presentable to CD8$^+$ CTLs from the endogenously expressed chimeric protein unless the GAr domain is deleted; conversely, insertion of the GAr domain into the EBNA3B sequence greatly reduces the efficiency with which EBNA3B epitopes are presented for CTL recognition. Significantly, all natural EBV isolates do show conservation of the EBNA1 GAr domain even though this domain is not required for the principal function of EBNA1, namely maintenance of the episomal viral genome in latently infected cells (31, 32). This may reflect the importance to the virus of rendering nonimmunogenic the one viral protein whose expression is likely to be essential for virus persistence.

The implication from the above work is that accessibility to the HLA class I–processing pathway could be one important factor determining the relative

immunogenicity of antigens for CTL responses. The conventional route of HLA class I processing involves the cleavage of cytosolically expressed protein, probably via the proteosome system, the delivery of derived peptides from the cytosol into the endoplasmic reticulum via the TAP1/TAP2 transporter complex, and the subsequent binding of high affinity peptides by nascent HLA molecules to form stable peptide/HLA complexes for presentation at the cell membrane (33, 34). The *cis*-acting protection from processing mediated by the GAr domain of EBNA1 would most easily be explained if it affected the protein's accessibility to the initial proteolytic cleavage, presumably by the proteosome. There is no reason to suspect that EBNA1 would be similarly protected from the HLA class II processing pathway; indeed CD4+ HLA class II–restricted CTLs have been detected that map to an EBNA1 epitope and that recognize LCL cells to which EBNA1 has been supplied as an exogenous antigen (35; R Khanna, DJ Moss, unpublished data). More surprisingly, however, rare CD8+ HLA class I–restricted CTL responses to EBNA1 have now been detected in the memory of two virus-immune donors (N Blake, SP Lee, AB Rickinson, unpublished data), suggesting that the GAr-mediated protection from processing may on occasion be overridden.

A second unusual feature of antigen processing in the EBV system involves the subdominant target antigen LMP2. Thus, experiments in a TAP-negative LCL background show that, while EBNA3A, 3B, and 3C proteins are not presented for CTL recognition in this environment, some CTL epitopes within LMP2 are still presented (36, 37). This is only the second documented example of TAP-independent processing of an endogenously expressed viral protein; the first was HIV-cnv, a molecule that naturally translocates into the endoplasmic reticulum (ER), where it is thought to become exposed to ER proteases (38, 39). LMP2, by contrast, has short cytosolically located N- and C-termini and a central hydrophobic region composed of 12 tandemly arranged transmembrane domains with very little projection into the endoplasmic reticulum (1, 40, 41). The mechanism whereby epitopes situated within transmembrane domains can be presented in a TAP-independent manner remains to be determined. However, by analogy with topologically similar ER proteins (42–44), a fraction of newly synthesized LMP2 molecules may be subject to ubiquitin-mediated proteosomal cleavage after membrane insertion, and this may release fragments that can then access the ER lumen.

Epitope Choice

Many of the above EBV latent antigen-specific CTL responses have now been mapped to defined epitopes within the primary sequence of these proteins. A complete list of these epitopes, including several recently identified in the context of less common HLA alleles, is shown in Table 1. This serves to illustrate the concentration of HLA class I epitopes in the EBNA3A, 3B, 3C proteins

Table 1   EBV-encoded CTL Epitopes

EBV antigen	Epitope co-ordinates	Epitope sequence	HLA restriction Class I	HLA restriction Class II	EBV type specificity	References
Latent cycle antigens						
EBNA1	407–417	HPVGEADYFEY	B35.01		nt	unp.
	515–527	TSLYNLRRGTALA		DR1	Type 1 & 2	35
EBNA2	42–51	DTPLIPLTIF	?A2/B51		Type 1	46
	280–290	TVFYNIPPMPL		DQ2	Type 1	unp.
EBNA3A	158–166	QAKWRLQTL	B8		Type 1	25
	176–184	AYSSWMYSY	A30.02		Type 1 & 2	70
	246–253	RYSIFFDY	A24		Type 1	25
	325–333	FLRGRAYGL	B8		Type 1	24
	379–387	RPPIFIRRL	B7		Type 1	23
	406–414	LEKARGSTY	B62		Type 1	unp.
	450–458	HLAAQGMAY	?		Type 1	25
	458–466	YPLHEQHGM	B35.01		Type 1	25
	491–499	VFSDGRVAC	A29		Type 1	unp.
	502–510	VPAPAGPIV	B7		Type 1	unp.
	596–604	SVRDRLARL	A2		Type 1 & 2	25
	603–611	RLRAEAQVK	A3		Type 1 & 2	21
EBNA3B	101–115	[NPTQAPVIQLVHAVY]	A11		Type 1	22
	149–157	HRCQAIRKK	B27.05		nt	unp.
	217–225	TYSAGIVQI	A24.02		Type 1	unp.
	244–254	RRARSLSAERY	B27.02		Type 1	unp.
	399–408	AVFDRKSDAK	A11		Type 1	22
	416–424	IVTDFSVIK	A11		Type 1	23
	481–495	[LPGPQVTAVLLHEES]	A11		Type 1	22
	488–496	AVLLHEESM	B35.01		Type 1	unp.
	551–563	[DEPASTEPVHDQLL]	A11		Type 1	22
	657–666	VEITPYKPTW	B44		Type 1	unp.
	831–839	GQGGSPTAM	B62		Type 1	unp.
EBNA3C	163–171	EGGVGWRHW	B44.03		Type 1 & 2	120
	213–222	QNGALAINTF	B62		Type 2	28
	249–258	LRGKWQRRYR	B27.05		Type 1	45
	258–266	RRIYDLIEL	B27.02/.04/.05		Type 1	45
	271–278	HHIWQNLL	B39		Type 1 & 2	unp.
	281–290	EENLLDFVRF	B44.02		Type 1 & 2	26
	284–293	LLDFVRFMGV	A2.01		Type 1 & 2	28
	335–343	KEHVIQNAF	B44.02		Type 1	18
	343–351	FRKAQIQGL	B27.05		Type 1	unp.
	881–889	QPRAPIRPI	B7		Type 1 & 2	23
EBNA-LP		occasional responses identified, no epitopes defined				18
LMP1		occasional responses identified, no epitopes defined				18
LMP2	131–139	PYLFWLAAI	A23		Type 1 & 2	37
	200–208	IEDPPFNSL	B60		Type 1 & 2	29
	236–244	RRRWRRLTV	B27.04		Type 1 & 2	45
	329–337	LLWTLVVLL	A2.01		Type 1 & 2	29
	340–350	SSCSSCPLSKI	A11		Type 1 & 2	29
	419–427	TYGPVFMCL	A24		Type 1 & 2	29
	426–434	CLGGLLTMV	A2.01		Type 1 & 2	27
	442–451	VMSNTLLSAW	A25		Type 1 & 2	unp.
	453–461	LTAGFLIFL	A2.06		Type 1 & 2	29

(Continued)

Table 1 *(Continued)*

EBV antigen	Epitope co-ordinates	Epitope sequence	HLA restriction Class I	Class II	EBV type specificity	References
Lytic cycle antigens						
BZLF1	190–197	RAKFKQLL	B8		nt	74
	186–201	[RKCCRAKFKQLLQHYR]	C6		nt	74
BMLF1	265–273	KDTWLDARM	?		nt	73
	280–288	GLCTLVAML	A2.01		nt	73
	397–405	DEVEFLGHY	B18		nt	73
BMRF1	86–100	[FRNLAYGRTCVLGKE]	C3		nt	73
	268–276	YRSGIIAVV	C6		nt	73
BHRF1	171–189	AGLTLSLLVICSYLFISRG		DR2	Type 1	72

Note that, after allowing for internal repeats, the number of unique amino acid residues in each of the latent cycle antigens is
372 for EBNA1, 446 for EBNA2, 944 for EBNA3A, 938 for EBNA3B, 992 for EBNA3C, 109 for EBNA-LP, 359 for LMP1 and
497 for LMP2 (calculated from the sequence of the reference type 1 EBV strain, B95.8).
[ ] Bracketed sequences indicate that the minimal epitope is not yet defined.
nt—type specificity not tested but epitope identified using CTLs from type 1 EBV-infected donors.
unp.—unpublished data from authors' laboratories.

(vis-à-vis nonpreferred antigens such as EBNA1, EBNA2, EBNA-LP, and LMP1) and also draws attention to the increasing number of subdominant responses that map to epitopes in LMP2. Such a distribution cannot be wholly ascribed to differences in size between the eight coexpressed latent proteins (see Table 1 footnote), nor to any obvious differences in their levels of expression in latently infected cells (1).

It is clear that epitope choice is highly allele-specific. Thus, although many different HLA alleles direct responses toward the EBNA3A, 3B, 3C proteins, each allele presents a particular set of CTL epitopes. The only clear example of epitope sharing involves three closely related subtypes of the B27 allele (B27.02, .04, and .05), all of which present the same immunodominant epitope from EBNA3C (45). Even here, however, each subtype also presents a second epitope that is subtype-specific (J Brooks, AB Rickinson, unpublished data). Secondly, epitope choice is highly focused in that individual alleles rarely present more than two or three peptide epitopes, one of which tends to be immunodominant. Thus a strong HLA-restricting allele such as B8 induces a response to one particular immunodominant epitope (EBNA3A 325–333) and to one subdominant epitope (EBNA3A 158–166) in almost all B8-positive individuals (24, 25, 46; DJ Moss, unpublished data); likewise A11 induces a response to EBNA3B 416–424 as the immunodominant epitope and to EBNA3B 399–408 as the subdominant epitope in most A11-positive individuals (22).

Recent studies suggest that the immunodominance of one epitope over another restricted through the same allele reflects differences in their levels of representation as peptide/HLA complexes at the LCL cell surface (47). Thus, the

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EBNA3B 416–424 peptide was at least tenfold more abundant than EBNA3B 399–408 in extracts of A11-positive LCLs. Interestingly, this appeared to reflect the longer half-life of 416–424/A11 complexes when measured at the surface of living cells, even though the two types of complex were equally stable under a number of conditions in cell-free assays. This difference in representation at the membrane could also help to explain why CTLs to the 416–424 epitope kill naturally infected LCL targets much more efficiently than do CTLs to the 399–408 epitope, whereas these two types of effector cell can recognize peptide-loaded targets down to equally low concentrations of exogenous peptide (47). More generally, a number of in vitro–reactivated CTL clones that map to EBV epitopes in peptide sensitization assays and that can recognize target cells overexpressing the relevant EBV antigen from a vaccinia vector nevertheless do not kill the naturally infected LCL. This may again reflect the fact that, for such clones, the level of representation of the relevant peptide/HLA complex at the LCL surface is insufficient to trigger lysis (21).

Table 1 also indicates whether these CTL reactivities are type-specific or cross-reactive between type 1 and type 2 virus strains. Not unexpectedly, since the allelic forms of EBNA3A, 3B, and 3C differ at 16%, 20%, and 28% of their residues, respectively (48), many of the immunodominant CTL responses in type 1–infected individuals map to epitopes that are not conserved in type 2 viruses. Furthermore, the first studies to be conducted on type 2 virus–infected individuals have likewise detected responses to epitopes in EBNA3A and in EBNA3C that are type 2–specific (28; IS Misko, unpublished data). By comparison, relatively little sequence diversity is found in the LMP2 protein, and many of the CTLs to LMP2 epitopes appear to be cross-reactive between virus types (27, 29). As to variation among EBV strains of the same type, certain epitopes were found to be well conserved across a range of type 1 isolates, for example, those restricted through the B8 and B44 alleles (49, 50). Likewise the A11 epitopes EBNA3B 416–424 and 399–408 were conserved in most type 1 strains from Caucasian and African populations; interestingly, however, these sequences were altered in isolates from China and coastal Papua New Guinea (51, 52), two areas where the prevalence of the A11 allele in indigenous populations is extremely high. That the changes specifically involved anchor residues affecting peptide/A11 binding and resulted in a failure of CTL recognition (51, 52) strongly suggested that such A11 epitope-loss mutations had been selected by immune pressure in these particular communities. This remains an intriguing possibility, but more recent data suggest that Southeast Asian virus strains, though predominantly type 1, are clearly distinguishable from type 1 Caucasian/African isolates by means of sequence microheterogeneity at various sites in the EBNA3A, 3B, 3C genes (50, 53; R Khanna, DJ Moss, unpublished

data). Some of these lie outside known CTL epitope regions, whereas others alter the antigenicity of epitopes restricted through HLA-alleles that are not even represented in these Southeast Asian populations. Conversely, EBV isolates from isolated highland regions of Papua New Guinea, where the A11 allele frequency is very low, show the same spectrum of A11 epitope-loss mutations as do viruses from the above highly A11-positive populations (50). It may therefore be that these differences in A11 epitope sequences are coincidental markers of a particular geographically restricted viral genotype (2) rather than the specific product of CTL-mediated selection.

T Cell Receptor Usage

Another interesting feature of EBV-induced CTL memory that has recently begun to receive attention is its T cell receptor (TCR) usage. Rearrangements of the TCR α and β chain genes during T cell development provide a vast potential for diversity in TCR $\alpha\beta$ heterodimers, particularly in the hypervariable CDR3 region that is postulated to interact directly with peptide epitopes (54). It is therefore remarkable that in several B8-positive individuals, the B8-restricted response to the immunodominant EBNA3A 325–333 epitope was found to be largely composed of clones with the same highly conserved TCR $\alpha\beta$ structure (55). Such a level of conservation in TCR usage is significantly greater than has been seen within epitope-specific CTL responses in other human viral systems such as influenza or HIV (56–58). This raises the possibility that long-term antigenic challenge with EBV, a virus (unlike influenza or HIV) that is both persistent and genetically stable, may progressively select for those memory CTLs with maximal affinity/avidity for the relevant peptide/HLA complex (see next section).

It will be interesting, therefore, to determine the level of TCR focusing within memory CTL responses to other EBV epitopes. In this context, B8-restricted memory to the subdominant EBNA3A 158–166 epitope appears to be more heterogeneous in TCR usage than that described above for the immunodominant EBNA3A 325–333 response (SL Silins, DJ Moss, unpublished data). However, the situation in relation to A11-restricted memory populations appears to be reversed, in that responses to the immunodominant EBNA3B 416–424 epitope are quite heterogeneous both within and between different A11-positive individuals, whereas responses to the subdominant EBNA3B 399–408 epitope tend to use sets of highly related TCRs (59). These authors propose that epitopes such as EBNA3B 399–408, which are present in limiting amounts on the LCL membrane (47), will tend to induce more focused responses involving only those CTLs whose receptors show high affinity/avidity for the peptide/HLA complex.

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Further analysis of the B8-restricted response to the immunodominant EBNA3A 325–333 epitope showed that all memory CTL clones with the highly conserved TCR structure also mediated cross-reactive lysis of EBV-positive or EBV-negative target cells expressing the B44.02 alloantigen (60). In fact, such is the level of this EBV epitope-specific memory in B8-positive virus carriers that their alloreactive responses to B44.02 in conventional mixed lymphocyte culture are dominated by CTLs from virus-specific memory with the relevant highly conserved TCR. This graphically illustrates how a prior history of infection with an immunogenic virus such as EBV can influence an individual's level of responsiveness to an alloantigen; such mechanisms may underlie the observed clinical association between herpesvirus status and graft-vs-host disease in bone marrow transplant patients (61). The above cross-reactivity is presumably recognizing a complex of the B44.02 alloantigen and a naturally processed self-peptide; not surprisingly, therefore, T cells with this particular TCR are not detected within the EBV-induced memory CTL population in individuals with both the B8 and B44.02 alleles. Interestingly, however, such individuals do still make a response to the EBNA3A 325–333/B8 complex through a variety of different TCRs, indicating the levels of reserve strength that exist within the TCR repertoire in the response to a particular target structure (62). More recent work has shown that cross-reactivity within the EBNA3A 325–333 response in B8-positive individuals is not limited to B44.02 only, and that certain less frequent components of the response recognize either B14 or B35.01 as alloantigens. In the latter case, some evidence suggests that the alloantigen-associated self-peptide is derived from the liver-specific cytochrome p450 (SR Burrows, DJ Moss, unpublished data). This is a particularly intriguing example of molecular mimicry with potential relevance to the pathogenesis of autoimmune hepatitis, a liver disease with a suspected EBV association and a strong link to HLA-B8 (63).

PRIMARY CTL RESPONSES

The prospective study of IM patients represents potentially one of the best opportunities to monitor the primary human CTL response to a viral infection and to follow its subsequent establishment in T cell memory. Progress in this area has been relatively slow, however, because the expanded CD8$^+$ T cell population in IM blood was found to contain several cytotoxic components in ex vivo assays; in particular the presence of CTLs showing cross-reactive recognition of alloHLA molecules made it difficult to discern any HLA class I-restricted lysis of LCL targets and hence any clear indication of EBV specificity (64–67). The specificity of the response was further called into question by a report that certain Vβ subsets were consistently expanded within the T cell

population of IM patients, suggesting the involvement of a virus-coded or virus-induced superantigen as the stimulus (68; see also next section).

A number of these issues have recently been reexamined, and a clearer picture is now emerging. First, the CD8$^+$ population in IM does show significant expansions of up to 30% of cells from particular Vβ subsets; however, the evidence suggests that these are antigen-driven and do not constitute a superantigen-like response. Thus, different Vβ subsets are affected in different patients, and very significantly, these Vβ expansions tend to show a markedly oligoclonal rather than polyclonal pattern of TCR usage, often with a single TCR structure dominant (69). Second, IM effectors have now been screened directly for EBV antigen and epitope specificity in ex vivo cytotoxicity assays using the recombinant vaccinia vector and peptide approaches. These assays have detected unequivocally an EBV-specific CD8$^+$ CTL response in IM that again is skewed toward a limited range of epitope peptides, most of which are derived from the EBNA3A, 3B, 3C subset of latent proteins (70). However, the primary response is not simply an exaggerated version of that subsequently seen in T cell memory; thus, the relative frequencies of individual epitope specificities may alter with the shift from primary to memory, and occasionally a particular specificity may be lost altogether. Do the oligoclonal Vβ expansions seen in IM blood correspond to EBV-specific responses, and do these particular clones enter memory? Preliminary studies on B8-positive IM patients, where there was a detectable primary response to the EBNA3A 325–333 epitope, clearly show the expansion of T cells with the same highly conserved TCR rearrangement as that already identified for this epitope in the memory of healthy carriers (69, 71). In this case, therefore, clones with the relevant TCR are present within the primary response. Longer-term studies are needed to determine whether these are preferentially selected into memory at the expense of coresident clones recognizing the same epitope but via different TCR structures.

Another interesting feature of the primary response is the presence of CTLs recognizing lytic cycle as opposed to latent cycle antigens. The first such reactivity to be described mapped to an epitope in the BHRF1 protein (an early lytic cycle antigen) but was unusual in being HLA class II–restricted and mediated by a rare CD4$^+$ CD8$^+$ T cell (72). It is now clear, however, that CD8$^+$ HLA class I–restricted CTLs to immediate early or early lytic cycle antigens are frequently detectable in the blood of IM patients. The best example to date is a B8-restricted response, directed against an epitope in the immediate early protein BZLF1, which in some patients may be even more abundant than the response to the immunodominant latent cycle epitopes (73). This BZLF1-specific reactivity was actually first detected in the memory of virus carriers following in vitro stimulation with a BZLF1 peptide pool (74). Identifying the full range of lytic antigen-specific reactivities in memory may well require

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new experimental approaches because in most cases the LCLs conventionally used as stimulators of memory CTL responses in vitro contain very few lytically infected cells (1). The fact that CTL responses to immediate early and some early proteins do exist in vivo suggests that antigen-presenting function is conserved in at least some cells in the early stages of the virus replicative cycle.

IMMUNOBIOLOGY OF EBV PERSISTENCE

EBV appears to have acquired a number of unique functions that enhance its ability to colonize the B cell system in the period immediately following viral transmission, to persist as a latent infection within the B cell pool in the face of CTL surveillance, and finally to reactivate from that latently infected B cell pool into lytic cycle, thereby maintaining oropharyngeal shedding by the virus-carrying host (2).

Successful infection of a new host critically depends on the ability of the virus to access the B cell system and to amplify the load of virus-infected B cells rapidly in the short period before the CTL response comes into play. Several features of the virus contribute to that end. One is the ability of the viral envelope glycoprotein gp340 to bind to the cell surface complement receptor molecule CR2 (75, 76), an interaction that mimics aspects of C3d complement binding (77) and that targets the virus specifically to the B cell lineage. Thereafter, the virus is able both to drive the proliferation of infected cells and to enhance their expression of cell survival signals such as Bcl2 through adopting an LCL-like program of full latent gene expression (4, 78). The main source of virus involved in this early colonization of the B cell system will be virions produced from local replicative lesions in the oropharynx. In this context one of the viral genes expressed during the late phase of the lytic cycle is BCRF1, a viral homologue of the cellular IL10 gene (79, 80), and at least two biological activities of this virally encoded cytokine may work to enhance successful infection of the B cell system. First, in studies in vitro, viral IL10 can promote the efficiency of virus-induced transformation through its capacity to augment early B cell proliferation (81, 82); second, though evidence conflicts on this point (83; and AD Stuart, M Mackett, personal communication), viral IL10 may serve to impede the local generation of CTL responses either to lytically or to latently infected cells (84). Very recent studies also highlight another mechanism whereby lytically infected cells may enhance the initial stages of growth transformation in adjacent B cells. This extrapolates from the interesting in vitro finding that LCL cells, if induced into lytic cycle, can under certain conditions selectively elicit a superantigen-like response in autologous T cells of the $V\beta13$ subset. If this were indeed to occur in vivo in the natural setting of EBV replicative lesions, it is suggested

that cytokines derived from the $V\beta 13$ T cell infiltrate may enhance local B cell transformation events (85).

The initial expansion of latently infected growth-transformed B cells seen in IM patients is eventually brought under control, presumably by the primary virus-specific CTL response, and a rapid fall occurs in viral load (2). However, the virus is never eliminated from the B cell pool, and a key event in the establishment of virus persistence must be the reversion of some growth-transformed B cells back to a resting state (86) and the concomitant downregulation of many of the virus latent cycle antigens that constitute the major targets for CTL attack. The cellular and/or viral controls that achieve this switch are completely unknown, and the actual pattern(s) of viral protein expression thus produced can only be surmised from transcriptional studies (4, 87, 88). The pattern appears to include EBNA1, the virus genome maintenance protein (31, 32), and/or LMP2, a protein thought to be involved in stabilization of the latent state (89, 90). Of these, EBNA1 is protected from presentation to $CD8^+$ CTLs (30), while LMP2 may not be displayed efficiently for CTL detection if antigen processing functions are themselves downregulated in a resting B cell (91). What determines the virus's ability to reactivate from this latently infected reservoir into lytic cycle is again unknown, but because the cells involved are a mobile recirculating population, it is tempting to think that physiological signals, perhaps received by B cells when infiltrating mucosal surfaces, may be used as the trigger (2). The process of reactivation and entry into a program of lytic antigen expression should then render these cells once again susceptible to CTL control, this time mediated by lytic antigen-specific effectors. Such CTLs clearly are present in virus-infected individuals, and therefore the CTLs have the potential to control rates of reactivation. However, it would not be surprising to find a mechanism whereby cells in EBV lytic cycle might at some stage become protected from CTL recognition, given the examples of herpes simplex and cytomegaloviruses, both of which employ specific lytic cycle products to interfere with the HLA class I–processing pathway (92–94).

CTL CONTROL OF EBV-ASSOCIATED TUMORS

The association between EBV and an increasing range of human tumors has been reviewed in detail elsewhere (2). Here we focus on these malignancies specifically as targets for CTL recognition.

Immunoblastic Lymphoma

Immunoblastic lymphoma (sometimes called posttransplant lymphoproliferative disease) is a B cell tumor that occurs frequently in T cell immunocompromised patients (9). The realization that these tumors are composed of

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EBV-transformed LCL-like cells expressing the full spectrum of virus latent proteins (95–97) had two important implications. First, it showed that EBV can indeed be directly oncogenic in vivo; second, it strongly suggested that the outgrowth of EBV-transformed cells would be reversed by a restoration of CTL control. The latter has now been dramatically proven in clinical practice (98–100), well ahead of the laboratory reconstruction demonstrating CTL-mediated regression of LCL outgrowth in severe combined immunodeficient mice (101). Thus in bone marrow transplant patients, where the lymphomas that arise are of donor B cell origin, the adoptive transfer either of peripheral blood mononuclear cells (98) or, more importantly, of in vitro reactivated EBV-specific CTL preparations (99) from the donor can rapidly reverse tumor growth. Furthermore, adoptively transferred CTL preparations could also be used prophylactically to reduce the EBV load in the B cell system, thereby reducing the risk of lymphoma development (99, 100). An essentially similar strategy could also be applied to solid organ transplant programs except that here any emerging lymphomas would be of recipient B cell origin and would need to be targeted by in vitro reactivated recipient CTLs; where necessary, such CTL preparations could be made from the patient's lymphocytes cryopreserved before transplantation.

Burkitt's Lymphoma

Burkitt's lymphoma (BL) is an unusual childhood tumor that in its high incidence endemic form is consistently EBV-positive (2). In marked contrast to matching LCLs, those BL cell lines that retained the original tumor cell phenotype were essentially nonimmunogenic in vitro, unable to stimulate either EBV-specific or even alloreactive T cell responses (102); furthermore, such cell lines were clearly not recognized by LCL-stimulated preparations of EBV-specific CTLs (103). The latter result was not due to any inherent resistance to cytolysis because the same lines were killed by some allospecific effectors (104) and could also be sensitized to EBV-specific lysis by exogenous loading of a relevant epitope peptide (105).

Several coincident features of BL cells appear to contribute to this immunologically silent phenotype. The inability to stimulate CTL responses in vitro probably reflects the absence or very low expression of ancillary molecules on the BL cell surface, in particular ICAM1 and LFA3 (106), which mediate adhesion to T cells via the LFA1 and CD2 pathways, respectively, and B7.1 and B7.2 (107; AB Rickinson, unpublished data) which deliver costimulatory signals to T cells via CD28 ligation (108). Interestingly, none of these aspects of the BL surface phenotype is a bar to CTL recognition per se, providing the CTL is preactivated and its relevant target structure is presented at the cell surface; in such circumstances the effector:target interaction appears to be

stabilized through ancillary adhesions involving ICAM2, an alternative LFA1 ligand constitutively expressed by BL cells (105).

The inability of EBV-specific CTLs to recognize BL targets reflects at least two additional features of this tumor. One is the well-documented downregulation of the viral genome such that only a single viral protein, EBNA1, is detectably expressed in BL cells (109). The second is a more recently discovered impairment of the HLA class I antigen presentation pathway itself. Thus the vaccinia-vectored expression of an immunogenic antigen (such as one of the EBNA3 proteins) within BL cells still does not sensitize the cells to specific CTL recognition, whereas lysis is observed if antigen processing and peptide transport are bypassed through expression of the minimal epitope peptide linked to an ER signal sequence (110). The above processing defect is associated with low expression of the TAP1/TAP2 genes, such that levels of the TAP proteins in BL cell extracts are often barely detectable compared to LCLs, and also with low expression of the HLA class I genes, such that cell surface HLA class I levels are <25% of LCL values (91, 110). Antigen processing function, as well as TAP and HLA expression, can be restored in several BL cell lines either by interferon γ treatment or, more interestingly, by vector-mediated expression of the EBV latent protein LMP1 (91). LMP1 is thought to be the major effector protein through which the virus drives resting B cells into growth (1) and, as such, must be central to the whole viral strategy for efficient amplification within the B cell system (2). Its coincident ability to upregulate the antigen processing pathway can also be seen as advantageous to the virus, however, because no benefit accrues if the virally transformed cells that initially disseminate the infection were immunologically silent and therefore able to grow out and kill the host.

It is intriguing that BL, a virally associated tumor, should display the classic immune escape phenotype. However, it is worth emphasizing that EBV-negative cases of this same malignancy (arising as rare childhood tumors outside endemic areas) show the same downregulation of ancillary molecules at the cell surface and the same deficiency in antigen processing (2, 91, 110). The immunologically silent nature of BL is therefore more likely to be a reflection of the particular progenitor cell from which the tumor arises (a germinal center B cell) (111) than of the tumor's viral association per se. Likewise the form of latency displayed by EBV in BL is not necessarily the product of immune selection but may reflect the virus's normal interaction with B cells at that particular stage of differentiation.

Nasopharyngeal Carcinoma (NPC) and Hodgkin's Disease (HD)

All cases of undifferentiated NPC both in high incidence (Southeast Asia) and low incidence areas are EBV genome-positive, as are at least 40% of cases of

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HD worldwide (2). Given that EBV-specific memory CTL responses can be reactivated in vitro from the blood of NPC and HD patients (112, 113), this again implies an ability of these tumors to grow in the face of CTL surveillance. Immunohistochemical analysis of fresh biopsies or of archival tissue in fact indicates that in both types of tumor the malignant cells express many of the adhesion and costimulatory molecules necessary for effective interactions with the T cell system and also display a TAP-positive, surface HLA class I-positive phenotype (114–117). This suggests that antigen processing functions are intact. Furthermore, recent studies with HD cell lines (albeit derived from EBV-negative cases of the disease) have also confirmed their ability to present vector-expressed viral antigens for CTL recognition in vitro (SP Lee, AB Rickinson, unpublished data).

A key feature of both NPC and HD is in fact the viral phenotype. Just as in BL, these tumors constitutively express EBNA1, but the remaining nuclear antigens (including the immunodominant EBNA3A, 3B, 3C proteins) are downregulated. Unlike BL, however, LMP1 is detectable in the malignant cells in at least a proportion of NPCs and in every EBV-positive case of HD, while the evidence from transcription studies suggests that both types of tumor are consistently LMP2-positive (2). With regard to these two virus-coded membrane proteins, there are occasional reports of CTL memory to LMP1 in virus-immune donors (18), but as yet the specificity of these responses has not been confirmed at the epitope level. By contrast, an increasing number of CTL epitopes are being defined in LMP2 for a range of HLA class I alleles such as A2.01, A2.06, A11, A24, and B60, which are relatively common in Caucasian and/or Southeast Asian populations (27, 29). Furthermore these epitopes are generally conserved among type 1 and type 2 EBV isolates worldwide (27, 29), another factor favoring their potential role as targets for a CTL-based tumor therapy. The inability of the CTL system to reject these potentially immunogenic tumors may be due in part to the inherent weakness of LMP2-specific responses, but may also reflect some local cytokine-mediated suppression of CTL activation in the vicinity of the tumor itself. An important observation in this regard was that EBV-specific CTLs could be reactivated from the blood of patients with EBV-positive HD but not from the tumor-infiltrating lymphocyte population (113). The possibility needs to be kept in mind that cytokines such as cellular IL10, known to be made by EBV-positive HD cells (118), may alter tumor immunogenicity (84, 119). Clearly a number of issues here deserve closer attention, particularly if in future one is to consider some form of CTL-based therapy for such malignancies. A key objective in this latter context would be to amplify only those components of the EBV-specific CTL response that are directed against viral antigens actually expressed in tumor cells (120).

VACCINE STRATEGIES

The ever-increasing range of EBV-associated disease and our better understanding of virus-induced immune responses together have led to renewed interest in the development of an effective vaccine. In western societies the principal aim of such a vaccine would be protection from infectious mononucleosis. In this context, it is noteworthy that only a proportion (up to 50%) of delayed primary infections present with clinical disease. Why this should be is not known; however, a high virus load (a large dose of orally transmitted virus and/or over-expansion of the virus-transformed B cell pool beyond a critical threshold) may be a critical determinant of disease risk (2). Therefore, a vaccine achieving even a modest reduction in EBV load during primary infection may be sufficient to avert clinical symptoms. Similarly, vaccination may also reduce the immediate risk of lymphoproliferative disease in patients such as pediatric liver transplant recipients, who frequently sustain primary EBV infection from the transplanted organ or from associated blood transfusions (9). In contrast, EBV-associated tumors such as BL, NPC, and HD arise in patients years after their primary infection, and protection from these longer-term consequences would require a vaccine that ideally conferred sterile immunity and prevented the establishment of the carrier state.

Two broad approaches to EBV vaccine development are currently being considered. The first seeks to exploit the major envelope glycoprotein gp340 as the immunogen, a strategy based initially upon the observation that this glycoprotein is the principal target of the virus-neutralizing antibody response (5, 6). Various formulations of gp340 either presented as subunit antigen or expressed from recombinant viral vectors are protective against EBV-induced B cell lymphoma in the tamarin (121); the mechanism of protection in this animal model is not yet clear but appears to be due to a combination of antibody and cell-mediated responses, with recent results suggesting a role for specific CTLs (122). Encouraged by the success of these animal experiments, a first vaccine trial has been conducted in China using a live vaccinia/gp340 recombinant. Sixteen months after vaccination, all children in the control group had become EBV-infected in the normal way [detected by antibody responses to the virus capsid antigen (VCA)], whereas 6/9 vaccinated children remained anti-VCA antibody negative (123). This result, if confirmed, is important because it implies that in some vaccinees the neutralizing antibody and/or cellular responses to gp340 had indeed induced sterile immunity, at least in the short term.

An alternative approach to vaccine development is based on the induction of EBV-specific CTL immunity. Here the aim is not to prevent primary infection per se but to limit those events occurring immediately postinfection, namely virus replication in the oropharynx and/or the expansion of virus-transformed

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B cells within the lymphoid pool. As reviewed in more detail elsewhere (124), any CTL-based vaccine strategy will likely aim toward immunization with combinations of defined epitopes. A first step toward this goal is to use formulations of synthetic peptides that mimic immunodominant epitopes known to be recognized by the natural virus-induced CTL response. Indeed a human phase 1 trial is currently underway in one of our laboratories (DJ Moss) using the HLA-B8-restricted epitope sequence FLRGRAYGL (EBNA3A 325–333) formulated in the water-in-oil emulsion, Montanide ISA 720, and tetanus toxoid as helper (124). This vaccine formulation is based on principles established in the murine cytomegalovirus model, where protection from virus challenge could be demonstrated following administration of a single immunizing dose of an immunodominant CTL epitope peptide (125). Obviously one of the major obstacles of any peptide epitope approach to vaccination in humans is HLA polymorphism because epitope choice is allele-specific. However, this obstacle might be overcome using appropriate mixtures of synthetic epitope peptides or by constructing vectors to express polypeptides in which the relevant epitope sequences are linearly joined together. Somewhat unexpectedly, when such an EBV polyepitope sequence was expressed within cells from a recombinant vaccinia vector, all of the constituent epitopes were efficiently presented for CTL recognition (126), indicating the potential of this approach as a vaccine strategy. More recently, work in a murine model has shown that each of several CTL epitopes combined in a polyepitope construct was capable of eliciting a CTL response in vivo and could protect the animals from subsequent challenge (127). In the longer term, it may be possible to combine the gp340-based and the CTL epitope-based approaches to EBV vaccination by generating a chimeric protein that fuses the important immunogenic domains of gp340 with a CTL polyepitope sequence.

FUTURE DIRECTIONS

EBV is a complex virus that can infect at least two different cell types in vivo and, depending upon the identity of the target cell and its situation, can either initiate lytic infection or establish one of several different forms of latency, each involving a different program of viral antigen expression. Different arms of the immune response are therefore likely to exercise control at different points in the viral life cycle. As described here, recent work has concentrated on one particular facet of host immunity, the HLA class I–restricted CTL response to latently infected growth-transformed B cells, at the expense of other mechanisms that, though less accessible experimentally, may nevertheless be equally important. Thus HLA class II–restricted CTL responses to EBV are detectable, but their antigenic specificities are at present poorly defined (15). Also very little is yet known about CD4$^+$ proliferative T cell responses, apart from some

preliminary studies with individual lytic cycle antigens (128–130); yet these could play an important cytokine-mediated role in vivo. The question of CTL control over lytic cycle infection is just beginning to receive long-overdue attention, and as yet little is known about the identity of the immunodominant lytic cycle antigens or about the antigen-presenting function of lytically infected cells.

It is now two decades since the atypical lymphocytes in IM blood were identified as reactive T cells (64), yet the cellular response to primary EBV infection and its relationship to disease pathogenesis are still poorly understood. Closer examination of IM T cell populations may reveal novel virus-induced reactivities that serve either to curtail or to prolong the disease process. Some of these reactivities may well not be EBV-specific, because primary infection with other agents such as HIV or cytomegalovirus can also sometimes induce a mononucleosis-like syndrome, and the cellular response may include common immunopathological elements in each case. In rare individuals, especially young boys with the X-linked lymphoproliferative (XLP) syndrome, primary infection induces a cellular response that is ostensibly similar to that seen in classical IM, yet the disease progresses to a fatal conclusion, culminating in what appears to be a cytokine-mediated destruction of hemopoietic tissues (2, 131). The awaited cloning of the XLP gene could yet provide important clues to the pathogenesis of IM itself. Another direction for future work again relates to EBV-induced immunopathology, but this time to the possibility that damage may be mediated by the virus-specific CTL response itself. Recently discovered examples of molecular mimicry between cognate EBV peptide/HLA target structures and certain self-peptide/HLA complexes (60; SR Burrows, DJ Moss, unpublished data) not only have implications in the context of allograft rejection but also may be very important in the pathogenesis of particular autoimmune diseases (125, 132, 133).

It is likely that EBV will remain one of the principal paradigms for study of the induction and maturation of human CTL responses to viral infection, in particular persistent infection. The differential immunogenicity apparent among latent cycle antigens needs to be examined in the context of other (noncaucasian) HLA backgrounds and of other viral strains, especially type 2 strains in which the EBNA3A, 3B, 3C proteins are antigenically distinct from their type 1 counterparts. If the hierarchy of immunodominance observed to date really is a general phenomenon, what can it reveal about the biochemistry of antigen processing in LCL cells? More specifically, how complete is the protection from processing enjoyed by the EBNA1 protein, and what is the mechanism whereby a glycine-alanine copolymer domain provides such protection? If, as now seems likely, EBNA1-specific HLA class I–restricted CTLs are detectable in at least some virus-immune donors, how were they elicited? Are EBV-

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specific CTL responses actually induced by virus-transformed LCL-like cells
in vivo or by a different antigen-presenting cell population?

Questioning the in vivo relevance of in vitro findings is a recurrent theme in
the EBV literature, not least in the field of immunology. More than anything,
this reflects the absence of a good animal model that mimics natural EBV
infection and persistence more closely than do the artificial situations produced
on experimental infection of certain New World primate species (121, 134). If
such a model existed, one could hope to dissect the different components of
immune responsiveness by cell depletion experiments and to look at the relative
importance of these different components in primary versus persistent infection.
In this context it is very interesting that many species of Old World primate
actually carry their own B lymphotropic herpesviruses that are closely related
to EBV in terms of genome structure (1), that have B cell growth transforming
ability, and that are likewise kept in check by immune T cell surveillance
in vivo (135). Not only does their existence have important implications for
our understanding of the evolution of EBV, it also opens up the possibility of
establishing tractable models that would recapitulate all the essential features
of EBV infection in humans.

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Quantification of antigen specific CD8+ T cells using an ELISPOT assay

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Abstract

An ELISPOT assay to detect and determine the number of antigen specific CD8+ T cells was standardized using cloned murine CD8+ T cells specific for the epitope SYVPSAEQI of a rodent malaria antigen. This assay is based on the detection of IFN-γ secretion by single cells after their stimulation with antigen. The interferon secretion is visualized as spots revealed by using enzyme labeled anti-IFN-γ monoclonal antibodies. Using known numbers of cloned murine CD8+ T cells it was determined that the assay detects 80–95% of these CD8+ T cells. The optimal culture conditions for the stimulation of the CD8+ T cells were determined and the antigen concentration, number of antigen presenting cells and supplement of growth factors required to perform the assay were defined. This ELISPOT assay can be performed with spleen cells from immunized mice, and provide the precise number of antigen specific CD8+ T cells present in mixed lymphocyte populations. This method is more sensitive than the chromium-51 release assay, and much simpler than the conventional precursor frequency analysis, providing the number of antigen specific CD8+ T cells in 36–48 h.

Keywords: CD8+ T cell; ELISPOT; IFN-γ; Synthetic peptide; Recombinant vaccinia virus; Malaria

1. Introduction

CD8+ T lymphocytes mediate one of the most important effector mechanisms of immunity against intracellular pathogens such as viruses, bacteria and parasites. These T cells recognize small peptides, derived from microbial antigens, which are presented on the surface of infected cells by class I MHC molecules.

Investigations on the mode of induction, and persistence of antigen specific CD8+ T cells, have been severely hampered by the lack of a simple assay capable of providing reliable quantitative data on these T cells. A widely used method for the detection and measurement of cytotoxic

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Abbreviations: CS, circumsporozoite; PYCS, recombinant vaccinia virus; IFN-γ, interferon-γ; MHC, major histocompatibility complex; DMEM, Dulbecco's modified eagle medium; PBS/T, phosphate-buffered saline containing 0.05% Tween 20; APC, antigen presenting cell.

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CD8$^+$ T cell activity is the ^{51}Cr release assay (Grabstein and Chen, 1980). This assay provides semi-quantitative data and is most useful for the detection of cytotoxic T cells. However, the ^{51}Cr release assay is inadequate for studies which require the determination of the number of antigen-specific CD8$^+$ T cells in a mixed lymphoid cell population. The best assay currently in use for such quantitative studies is the precursor frequency analysis, using the limiting dilution technique (Taswell, 1981). While it is generally accepted that this is an excellent method, its use is severely limited by the fact that it is very time consuming and laborious, and therefore unsuitable for studies which require the simultaneous comparison of several sets of experimental data.

We report here the standardization of an alternative method to determine the number of antigen specific CD8$^+$ T cells. This method was adapted from the ELISPOT assay described for the detection of mitogen activated CD4$^+$ T cells (Taguchi et al., 1990) and is based on the visualization of IFN-γ secretion by single CD8$^+$ T cells after their in vitro stimulation with antigen. The IFN-γ secretion by individual cells is visualized as spots revealed by using enzyme labeled anti-IFN-γ monoclonal antibodies. Using cloned murine CD8$^+$ T cells, which recognize a well defined epitope, we determined the optimal conditions for in vitro culture, antigen stimulation and detection of IFN-γ secretion by CD8$^+$ T cells. This assay is a rapid, highly sensitive method providing a quantitative assessment of the number of activated antigen specific CD8$^+$ cells. It differs in some important methodological aspects from the originally described ELISPOT assay used for the enumeration of IFN-γ secreting CD4$^+$ T cells (Taguchi et al., 1990; Xu-Amano et al., 1992).

2. Material and methods

2.1. Cells and cell culture medium

The CD8$^-$ T cell clone YA26, derived from a BALB/c (H-2^d) mouse immunized with *Plasmodium yoelii* sporozoites, was used throughout these experiments. The generation, the antigen specificity and the conditions for in vitro culture of this T cell clone have been described in detail elsewhere (Rodrigues et al., 1991,1992). Briefly, YA26 cells were cultured in DMEM high glucose (Gibco) medium, supplemented with 10% fetal calf serum (FCS), 10 mM Hepes, 2% EL-4 cell supernatant, 10^{-5} M 2-mercaptoethanol (2-ME) and 2 mM L-glutamine. P815 cells (H-2^d) were used as target cells for both the ^{51}Cr release and the ELISPOT assays. The EL-4 supernatant used as source of interleukins was obtained by stimulating EL-4 cells with PMA. It was used at a final concentration of 2% which contains 30 U of IL-2/ml.

2.2. ELISPOT assay for the detection of IFN-γ producing YA26 cells

96-well nitrocellulose plates (Mililiter HA, Millipore, Bedford, MA) were coated with 10 μg/ml of the anti-mouse IFN-γ mAb R4 (from the American Type Culture Collection, Bethesda, MD), in 75 μl of phosphate-buffered saline (PBS). After overnight incubation at room temperature, the wells were repeatedly washed with culture medium and 100 μl of DMEM high glucose, containing 10% FCS and 10 mM Hepes, were added to each well for 1 h at 37°C. Known numbers of CD8$^+$ T cells of the YA26 clone were then suspended in culture medium and placed into the antibody-coated wells.

P815 cells were incubated with the synthetic peptide SYVPSAEQI, for 1 h at 37°C. After repeated washings with culture medium, these peptide-pulsed P815 cells were irradiated, and added to the ELISPOT wells. P815 cells, not pulsed with peptide, were used as controls of antigen independent IFN-γ secretion.

After incubation at 37°C and 5% CO$_2$ for 24–28 h, these plates were extensively washed with PBS containing 0.05% of Tween 20 (PBS/T). The wells were then incubated with a solution of 5 μg/ml of biotinylated anti-mouse IFN-γ mAb XMG1.2 (Pharmingen, CA) in PBS/T. The plates were incubated overnight at 4°C, washed with PBS/T and then 100 μl of peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories, KPL, Gaithesburg, MD), at a 1/800 dilution in

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PBS/T, were added to each well. After 1 h incubation the wells were washed twice with PBS/T and twice with PBS. The spots were developed by adding 50 mM Tris-HCl at pH 7.5, containing 1 mg/ml of the substrate 3,3'-diaminobenzidine-tetra-hydrochloride dihydrate (DAB), and 5 μl/10 ml of 30% H_2O_2 to each well. After 10–15 min the number of spots were determined with the aid of a stereomicroscope.

2.3. Chromium release assay

The chromium release assay was performed as described elsewhere (Rodrigues et al., 1991). Briefly, 10^6 P815 cells were labeled with 250 μCi ^{51}Cr in FCS, for 1 h at 37°C. YA26 cells were incubated, at different ratios, with 2.5×10^3 ^{51}Cr labeled P815 cells per well, in the presence or absence of 10^{-7} M peptide SYVPSAEQI. After 5 h of incubation at 37°C, the supernatants were collected with the aid of a semi-automatic harvester (Skatron, Sterling, VA), and the number of counts determined. The percentage of specific lysis was calculated as previously described (Rodrigues et al., 1991).

2.4. Immunization of mice with a recombinant vaccinia virus expressing the SYVPSAEQI epitope and determination of splenic antigen specific CD8^{+} T cells by the ELISPOT assay

In experiments designed to determine the number of CD8^{+} epitope-specific IFN-γ secreting T cells, groups of three BALB/c mice were immunized i.p. with 5×10^7 pfu of recombinant vaccinia virus expressing the P. yoelii CS protein (PYCS) (Li et al., 1993). 11 days after the immunization, the mice were killed, their spleens removed, and the number of epitope specific IFN-γ producing cells determined. We used freshly isolated spleen cells and also further in vitro stimulated spleen cells. Different concentrations of freshly isolated splenocytes, starting at 5×10^5 cells/well, were screened for antigen specific CD8^{+} cells using the standardized ELISPOT assay. In addition, 5×10^7 spleen cells of the same pool were expanded in vitro for 6 days with 3×10^6 P815 cells pulsed with 10^{-7} M peptide SYVPSAEQI. After this expansion, a series of different concentrations of these splenocytes,

starting with 1.25×10^5 cells/well, were analyzed by the standardized ELISPOT assay.

2.5. Precursor frequency analysis

BALB/c mice were immunized with 5×10^7 pfu of recombinant vaccinia PYCS, as described for previous experiments. 30 days after immunization, spleen cells were used to perform the ELISPOT assay and the precursor frequency analysis. The ELISPOT was performed following the procedures described for the previous experiments. The precursor frequency analysis was performed as previously described (Ceredig et al., 1987). Briefly, 0, 150, 300, 600, 1200 and 2400 spleen cells from immunized mice were cultured with 7×10^5 irradiated syngeneic normal spleen cells and 2×10^5 irradiated peptide-pulsed P815 target cells, in 96-well flat-bottom culture plates. As culture medium, we used DMEM supplemented with 10% FCS and 2.5% EL-4 supernatant. At day 8, the contents from each well were split into 96-well round bottom plates to test the cytotoxic activity against peptide-pulsed ^{51}Cr labeled P815 target cells. As control, we used radiolabeled target cells not pulsed with peptide. After 6 h the ^{51}Cr release was measured and cultures were considered positive if the level of specific ^{51}Cr release was greater, by three standard deviations, than the release detected from radiolabeled target cells incubated without spleen cells. The frequency of antigen specific CD8^{+} T cells was calculated as previously described (Quintans and Lefkovits, 1973).

3. Results

3.1. Sensitivity of detection of cloned CD8^{+} T cells by the ELISPOT and by the ^{51}Cr release assay

A murine CD8^{+} T cell clone, YA26, which recognizes a class I MHC restricted epitope (SYVPSAEQI), of the CS protein of P. yoelii (Rodrigues et al., 1991), was used to determine the level of sensitivity of detection of antigen specific CD8^{+} T cells by the ELISPOT and the ^{51}Cr assay.

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Fig. 1. *Top panel:* detection of IFN-γ secretion following antigen activation of cloned CD8^{+} T cells (YA26). Variable numbers of cells of the YA26 clone were incubated with 10⁵ P815 cells pulsed with SYVPSAEQI peptide, at a final concentration of 10^{-6} M. After 28 h of incubation at 37°C and 5% CO_2, the wells were washed and the presence of IFN-γ spots revealed as described in the materials and methods section. To one series of these wells a 2% EL-4 supernatant was added (●), whereas in an otherwise identical series, the EL-4 supernatant was ommitted (▽). Each point represents the mean of triplicates ± SE. *Middle panel:* antigen specificity of the IFN-γ spots. 300 YA26 cells were incubated with P815 target cells, as described for the *top panel.* The IFN-γ spots were clearly visible when the YA26 cells were incubated with target cells pulsed with the SYVPSAEQI peptide (*B*), while they are absent when target cells without peptide were used (*A*). *Bottom panel:* ⁵¹Cr release assay using YA26 T cells and P815 target cells. Variable numbers of the T cell clone YA26 were incubated for 5 h with 2.5×10^3 radiolabelled P815 target cells, which had either not been pulsed (O) or were pulsed with peptide (●). Each point represents the mean of triplicates.

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For this purpose known numbers of cloned CD8$^+$ T cells were incubated in nitrocellulose-based culture wells (ELISPOT wells), with antigen (peptide)-coated P815 target cells. These wells had earlier been coated with mAb to murine IFN-γ, as described (Taguchi et al., 1990). After 24–28 h of incubation at 37°C and 5% CO$_2$, the wells were washed, incubated with biotinylated anti IFN-γ antibody, followed by the addition of avidin-peroxidase and a peroxidase substrate.

As seen in Fig. 1 (top panel), we obtained an excellent correlation between the number of cloned CD8$^+$ T cells placed into each well and the number of IFN-γ spots. In fact, this assay detected between 80 and 95% of the cells of a given well. We found that addition of an EL-4 supernatant to the culture medium, to provide a supplement of interleukins was important to ensure optimal production of IFN-γ by the CD8$^+$ T cells. In the absence of the EL-4 supernatant, the number of spots, we detected, accounted for only 50–60% of the total number of the CD8$^+$ cells of each well.

The IFN-γ spots, detected by this assay, were clearly antigen specific, since few or no spots

Fig. 2. Number of antigen-specific IFN-γ producing CD8$^+$ T cells detected antigenic stimulation in vitro for either for 1 or 7 days. YA26 CD8$^+$ T cells were stimulated with antigen for 1 day and then transferred to ELISPOT wells, immediately after mixing them with P815 target cells pulsed with (●) or without peptide (▼). In another series, YA26 CD8$^-$ T cells were stimulated with antigen for 7 days and then placed into ELISPOT wells immediately after adding P815 cells pulsed with (▽) or without SYVPSAEQI peptide (□). Each point represents the mean of triplicates ± SE.

were observed upon incubation of the CD8$^+$ T cells with P815 target cells, in the absence of the peptide (Fig. 1 (middle panel)). Thus, the presence of antigen appears to be essential to trigger IFN-γ production, while the interleukins provided by the EL-4 supernatant, though not strictly necessary, significantly improve production of this cytokine.

Comparing the sensitivity of the ELISPOT (Fig. 1 (top panel)) with the ^{51}Cr release assay (Fig. 1 (bottom panel)), it became clear that under optimal conditions the ELISPOT can detect small numbers of activated CD8$^+$ T cells, namely as little as 20–30 antigen-specific CD8$^+$ lymphocytes per well. In contrast, 10^3 or more of these CD8$^+$ cells were required to produce a clear signal in the ^{51}Cr release assay. Most importantly, the ELISPOT allows the determination of the number of antigen-specific cells, which can not be done by the use of the chromium-release assay.

3.2. Optimal conditions for the antigen stimulation of CD8$^-$ cells in the ELISPOT assay

There are significant methodological differences between the earlier described ELISPOT assay for the detection of IFN-γ secreting T cells (Taguchi et al., 1990; Xu-Amano et al., 1992; Di Fabio et al., 1994) and the ELISPOT we standardized for the detection of CD8$^+$ T cells. In the previously described ELISPOT assays, the T cells are first stimulated with mitogen or antigen in standard culture plates, during 1 day or more, before being placed in the ELISPOT wells. In contrast, we found that antigen-specific IFN-γ secreting CD8$^+$ T cells can only be detected if they are placed in the ELISPOT wells, immediately after being mixed with antigen-coated target cells, i.e., without pre-incubation in a standard culture plate. As shown in Fig. 2, if CD8$^+$ T cells are stimulated with antigen 1 or 7 days before being placed in the ELISPOT wells, the γ-interferon spots can not be detected, unless these cells are mixed with antigen coated target cells just before being placed in the ELISPOT wells.

The number of detectable spots produced by antigen specific CD8$^+$ T cells is strictly dependent on the concentration of antigen used to

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Fig. 3. Optimal concentrations of peptide SYVPSAEQI for the detection of CD8+ YA26 cells by the ELISPOT assay. Variable numbers of YA26 cells per well were incubated with 10^5 P815 cells pulsed with different concentrations of peptide. The peptide was used at concentrations of 10^{-6} M (●), 10^{-7} M (▽), 10^{-8} M (▼), 10^{-9} M (□) and 10^{-10} M (■). Each point represents the mean of triplicates ± SE.

Fig. 4. Effect of the number of antigen-presenting cells (P815) on the number of IFN-γ producing cells detected by the CD8+ ELISPOT assay. Twenty to 130 YA26 cells were incubated with 10^6 (●), 10^5 (▽), 10^4 (▼) or 10^3 (□) P815 cells pulsed with peptide SYVPSAEQI at a concentration of 10^{-6} M. Each point represents the mean of triplicates ± SE.

pulse the P815 cells. As shown in Fig. 3, a maximal number of interferon spots can be detected when target cells are pulsed with peptide concentrations ranging from 10^{-6} to 10^{-8} M. A significant decrease in the number of IFN-γ spots, or their absence, occured when peptide concentrations of 10^{-9} or 10^{-10} M were used, respectively.

3.3. Sensitive detection and enumeration of CD8+ antigen specific T cells

The number of antigen presenting cells (APC) used in the assay is also an important variable. Their optimal concentration appears to be 10^5 P815 cells per well. The use of either a larger or smaller number of target cells decreased considerably the number of IFN-γ spots (Fig. 4).

The type of antigen presenting cells used in the ELISPOT assay appears to affect only to a small degree the generation of IFN-γ spots. As seen in Fig. 5, the best APC for the activation of the Y26 CD8$^-$ T cells appears to be the P815 mastocytoma cell line. However, rather similar results were obtained when we used other types of APC such as the macrophage cell line J-774 (ATCC: TIB-67), or the B-cell lymphoma derived A20 cell line (ATCC: TIB-208).

3.4. Detection of antigen-specific CD8+ T cells in the spleens of immunized mice

With a view on the use of this assay for the detection of antigen-specific CD8+ T cells of immunized mice, we determined the possible influence that unfractionated spleen cells may have

Fig. 5. ELISPOT assay performed using different types of antigen presenting cells (APCs). 100 YA26 CD8+ T cells were incubated with 10^5 APC of different cell types (P815, A20 or J774) expressing the H-2K^d MHC molecules. With each cell line the assay was performed in the presence and absence of peptide. Each bar represents the mean of duplicates ± SE.

Fig. 6. The presence of naive spleen cells does not interfere with the sensitivity of the CD8⁺ ELISPOT assay. 100 YA26 cells plus 10⁵ P815 cells, pulsed with 10⁻⁶ M peptide, were incubated with various numbers of spleen cells obtained from normal BALB/c mice. Each point represents the mean of triplicates ± SE.

on this detection. A constant number of cloned CD8⁺ T cells and peptide-coated target cells were incubated with varying numbers of naive spleen cells. As can be seen in Fig. 6, the presence of variable, even very large numbers of

spleen cells did not affect the IFN-γ secretion by CD8⁺ T cells, in this assay.

We then used this ELISPOT to determine the number of antigen-specific CD8⁺ T cells in the spleen of mice immunized with a recombinant vaccinia virus expressing the epitope, SYVP-SAEQl in the context of the CS protein (Li et al., 1993). Eleven days after the immunization of mice with 5 × 10⁷ pfu of this recombinant vaccinia virus, their splenic lymphocytes were obtained and the number of epitope specific CD8⁺ splenic T cells was determined. We used both fresh spleen cells (immediate ELISPOT) and spleen cells after in vitro stimulation with antigen (ELISPOT after expansion).

The immediate ELISPOT assay, performed with freshly obtained spleen cells, revealed the presence of epitope-specific CD8⁺ T cells induced by the immunization with the recombinant viruses (Fig. 7 (left panel)). Most of the IFN-γ spots detected in the assay were antigen-specific, since only very few spots can be observed when these immune spleen cells were incubated with target cells in the absence of antigen. Furthermore, no antigen specific IFN-γ spots could be

Fig. 7. Detection of antigen specific CD8⁺ T cells in the spleen of mice immunized with vaccinia virus PYCS. 11 days after immunization of BALB/c mice with 5 × 10⁷ pfu of recombinant vaccinia virus PYCS, their spleen cells were obtained. A CD8⁺ ELISPOT assay was performed, either on the same day (left panel, immediate ELISPOT), or after 6 days in culture of these cells (right panel, expanded ELISPOT). For the expanded ELISPOT, 5x10⁷ spleen cells were cultured for 6 days in 10 ml DMEM of a high glucose medium containing EL-4 supernatant, together with 3x10⁶ P815 cells pulsed with 10⁻⁶ M peptide. The subsequent phase of these assays, conducted in the ELISPOT wells, was performed in the presence and in the absence of EL-4 supernatant, and in the presence or absence of peptide-pulsed target cells (specificity control). Each bar represents the mean of triplicates ± SE.

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detected upon immunization of mice with the wild type vaccinia virus (not shown). Not surprisingly, the chromium release assay performed with these freshly obtained spleen cells failed to reveal the presence of antigen-specific cytotoxic activity (data not shown). As expected, a greater number of antigen specific CD8+ T cells was detected when the ELISPOT was performed using the spleen cells 6 days after their in vitro stimulation with antigen (Fig. 7 (right panel)).

In agreement with earlier experiments, performed with cloned CD8+ T cells, we determined that the addition of 2% EL-4 supernatant to the spleen cells, improved considerably the detection of IFN-γ secreting cells. This improvement was particularly striking when fresh spleen cells were used in the assay (Fig. 7 (left panel). In the case of the spleen cells expanded in vitro by antigen stimulation, the situation was not as clear. As shown in Fig. 7 (right panel), the use of EL-4 supernatant increased appreciably the number of IFN-γ producing cells. However, the addition of the EL-4 supernatant also resulted in a significant increase in the number of cells which secreted IFN-γ in the absence of peptide, i.e., in a seemingly antigen-independent fashion.

Finally, we determined the number of antigen specific CD8+ T cells using both, the ELISPOT assay and the standard precursor frequency analysis using the limiting dilution technique. For this purpose, mice were immunized with the recombinant vaccinia virus and 30 days later their spleen cells were used to perform these assays. As seen in Fig. 8, the number of antigen specific CD8+ T cells detected by both assays was very similar. This result further validates the use of the ELISPOT assay for the quantification of antigen specific CD8+ T cells.

4. Discussion

Here we describe the standardization of a very sensitive, quantitative ELISPOT assay for the detection and determination of the number of antigen-specific CD8+ T cells. In order to perform this ELISPOT assay with the best possible results, we standardized it by using a clone (YA26) of CD8+ T cells of defined epitope specificity, so that a known number of these cells were placed in the ELISPOT wells. This permitted us to de-

Fig. 8. Quantification of antigen specific CD8+ T cells using the ELISPOT assay and the limiting dilution analysis. BALB/c mice were immunized with 5x10⁷ pfu of recombinant vaccinia virus PYCS expressing the SYVPSAEQI epitope. Thirty days after immunization the immune spleen cells were used to determine the number of IFN-γ producing antigen specific CD8+ T cells using the immediate ELISPOT assay and the standard cytotoxic T lymphocyte precursor frequency analysis (CTLp) using the limiting dilution technique. In both assays, the number of CD8+ T cells was determined in the presence and absence of the peptide SYVPSAEQI.

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termine precisely the sensitivity of this method, and to establish optimal conditions for the in vitro culture and antigen stimulation of these cells.

One of the steps that appears to be crucial for the detection of $CD8^+$ T cell derived IFN-γ spots is the need for mixing the $CD8^+$ T cells and peptide-coated target cells just prior to placing them in the ELISPOT wells, avoiding the cell transfer and consequent loss of secreted IFN-γ. This differs from the ELISPOT used for the detection of $CD4^+$ T cells, in which it is necessary to stimulate the immune T cells with antigen and APC one or more days before transferring these mixed cell population into the ELISPOT wells (Xu-Amano et al., 1992)

We identified additional variables which greatly affect the efficacy of the ELISPOT assay in detecting antigen-specific $CD8^+$ cells. Our results clearly demonstrate that addition of exogenous interleukins (EL-4 cell supernatant) to the culture medium, increases greatly the sensitivity of this assay. The mechanisms by which the interleukins enhance the functional activity of these cells remains to be determined. In this regard it is noteworthy that the absence of EL-4 supernatant during the time it takes to perform the ELISPOT, does not significantly impair the viability of the $CD8^+$ T cells (data not shown). The antigen concentration used to pulse the target cells, and the number of antigen presenting cells used in the assay, are also variables which influence appreciably the number of detectable IFN-γ spots.

In the context of applying the ELISPOT assay for the determination of the number of antigen specific $CD8^+$ T cells, it is necessary to establish experimental conditions which ensure that $CD8^+$ T cells are the only IFN-γ secreting cells being detected. In the case of clone YA26, which we used for the standardization, it is well established that the epitope SYVPSAEQI is recognized by $CD8^+$ T cells in the context of H-$2K^d$ class I MHC molecules, and that this sequence is not recognized by $CD4^+$ T cells (Romero et al., 1990; Rodrigues et al., 1994). However, when larger antigens or complex antigenic mixtures are used, it will be necessary to exclude the possibility of concomitant activation of both $CD4^+$ and $CD8^+$ T cells.

This problem can in part be circumvented by using antigen presenting cells, which express only class I not class II MHC molecules, such as the P815 mastocytoma cell line. As described in the methods section, these cells are extensively washed after 1 h of incubation with antigen, prior to incubation with the T cells. Under these conditions, it is expected that the antigen, introduced into the culture, is all bound to the P815 target cells.

Another alternative is to perform this assay using $CD4^+$ T cell depleted lymphoid cells. These $CD4^+$ depleted cell populations can be obtained by (a) in vivo treatment of the immunized mice with anti-CD4 antibodies, or (b) in vitro pre-incubation of the T cell preparations with anti-CD4 antibodies. In our system, as expected, neither the in vivo nor the in vitro depletion of $CD4^+$ T cells affected the detection of antigen-specific $CD8^+$ T cells. In sharp contrast, when we used $CD8^+$ depleted spleen cells the opposite occurred, i.e., IFN-γ spots could no longer be detected (Rodrigues et al., 1994).

The ELISPOT assay for the detection of antigen specific $CD8^+$ T cells, appears well suited for monitoring quantitatively various basic immunologic phenomena, such as the kinetics of induction of effector and of memory $CD8^+$ T cells, as well as the organ compartmentalization, homing and trafficking of these cells. This methodology is expected to have broad applications in vaccine development, particularly for the evaluation of the immunogenic properties of sub-unit vaccines designed to induce $CD8^+$ T cells against intracellular microbial pathogens. Recently we have used this assay to evaluate and compare the immunogenicity of various recombinant vaccinia and influenza viruses, expressing the SYVPSAEQI epitope in different structural contexts (Rodrigues et al., 1994).

In conclusion this ELISPOT assay has clear advantages over the standard chromium-release methodology since it is more sensitive, and permits the determination of the number of antigen-specific cells. It also compares favorably

with the standard frequency precursor analysis which uses the limiting dilution technique, because it is much simpler to perform, is less time consuming and less labor intensive, the results becoming available within 36–48 h.

Acknowledgements

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Phenotypic Analysis of Antigen-Specific T Lymphocytes

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Identification and characterization of antigen-specific T lymphocytes during the course of an immune response is tedious and indirect. To address this problem, the peptide–major histocompatability complex (MHC) ligand for a given population of T cells was multimerized to make soluble peptide-MHC tetramers. Tetramers of human lymphocyte antigen A2 that were complexed with two different human immunodeficiency virus (HIV)–derived peptides or with a peptide derived from influenza A matrix protein bound to peptide-specific cytotoxic T cells in vitro and to T cells from the blood of HIV-infected individuals. In general, tetramer binding correlated well with cytotoxicity assays. This approach should be useful in the analysis of T cells specific for infectious agents, tumors, and autoantigens.

Quantitative analyses of antigen-specific T cell populations have provided important information on the natural course of immune responses (1–3). Currently, the standard method for deriving frequency information is limiting dilution analysis (LDA) (3). However, this technique may significantly underestimate the number of specific T cells because it cannot detect cells that have no proliferative potential (4–6). Although flow cytometry provides a fast and direct method for enumerating cells expressing a particular antigen on their surface, detection of low-frequency populations of antigen-specific lymphocytes by staining with their cognate antigen has only been demonstrated for B lymphocytes, making use of the high affinity for antigen that many of these cells have (7). Antigen-specific T cells from normal, immunized mice have been identified and analyzed in a few systems, with T cell receptor V region antibodies as surrogate markers for antigen specificity (1, 8), but the more general approach of staining specific T cells with their ligand has failed because soluble peptide-MHC complexes have an inherently fast dissociation rate from the T cell antigen receptor (9).

Fig. 1. Staining by MHC-peptide tetramers correlates with peptide-dependent cytotoxicity. Flow cytometric analysis (16) of CD8+ T cells (17, 20, 30) from (A) clone 20 stained with A2-Pol (solid line) and A2-Gag (dotted line) tetramers, (C) HIV-Gag-specific CTL line 868 stained with A2-Gag (solid line) and A2-Pol (dotted line) tetramers, and (E) an HLA-A2–restricted influenza matrix peptide CTL line (PG-001), stained with A2-MP tetramers and sorted into A2-MP+ and A2-MP− populations, as indicated. Cytotoxicity assays with (B) clone 20 showed specific killing of autologous Epstein-Barr virus–transformed B cells pulsed with Pol peptide (closed squares) but not target cells without added peptide (closed circles). (D) The 868 Gag-specific CTL line killed cells pulsed with the Gag peptide (closed squares) but not target cells without added peptide (closed circles). (F) The sorted populations from (E) were assayed for killing of MP-pulsed target cells at an effector:target ratio of 1:1. At the same ratio, in cells not treated with peptide, no killing of target cells was seen.

Multimeric peptide-MHC complex should be able to bind more than one T receptor (TCR) on a specific T cell, thus have correspondingly slower dissociation rates, making the complexes more stable for use as an immunological stain. To engineer tetrameric peptide-MHC complexes, we first added a 15-amino acid substrate peptide for BirA-dependent biotinylation (10) to the COOH-terminus of human lymphocyte antigen (HLA) heavy chain. After folding the heavy-chain fusion protein in vitro in the presence of β2-microglobulin (β2M) and a specific peptide ligand (11), the purified MHC-peptide complex was biotinylated efficiently (~100%) on a single lysine within the substrate peptide (BSP) (12). Biotinylation at the COOH-terminus of the heavy chain also served to correctly orient the MHC molecule, as the site recognized by the TCR involves the NH2-terminal domains. Tetramers were produced by mixing biotinylated peptide-MHC complex with phycoerythrin-labeled deglycosylated

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...lip at a molar ratio of 4:1.

In HIV-infected HLA-A0201+ individuals, strong anti-HIV cytolytic T lymphocyte (CTL) responses are often observed that target one of two major HLA-A2–restricted HIV epitopes, Gag(77–85) (14) or reverse transcriptase(309–317) (15, 16). To test the utility of tetrameric peptide-MHC complexes as staining reagents, we prepared HLA-A2 tetramers with either the Gag or Pol peptide and used them to stain HLA-A2–restricted, HIV-specific CTL lines or clones (17, 18) (Fig. 1). The A2-Pol–specific clone 20 is stained by the A2-Pol tetramer but not the A2-Gag tetramer (Fig. 1A), whereas 10% of the cells in the uncloned Gag-specific CTL line 868 are stained by the Gag reagent, but no staining is observed with the Pol reagent (Fig. 1C). In each case, the presence of a positively staining population correlates with functional killing assays (19) (Fig. 1, B and D). To further demonstrate the generality and specificity of these reagents, we prepared a third HLA-A2 tetramer containing a peptide (MP) from the influenza A matrix protein M58–66 and used it to sort cells (PG-001) from an HLA-A2–MP-specific CTL line. Approximately 10% of the CD3+ cells in the line bound the A2-MP tetramer (Fig. 1E), and binding of the tetramer was completely inhibited by an antibody to TCR V$_\beta$17, the variable gene segment that dominates the HLA-A2–MP response (20). The line was sorted into CD3+A2-MP+ and CD3+A2-MP− fractions, which were then assayed for CTL activity at an effector:target ratio of 1:1. Significant killing activity was observed in the A2-MP+ fraction but not in the A2-MP− fraction (Fig. 1F).

Next, we tested the A2-Gag and A2-Pol tetramers for their ability to identify HIV-specific CD8+ cells present in freshly isolated peripheral blood mononuclear cells from HLA-A0201+, HIV-seropositive donors, and thereby quantitate them rapidly and directly. Fresh peripheral blood mononuclear cells from four HLA-A2+, HIV-seropositive patients were analyzed by three-color flow cytometry with the A2-Pol and A2-Gag tetramers (Fig. 2). Background staining levels were established by similar analyses of peripheral blood cells from HIV-seronegative, low-risk, HLA-A0201+ donors. To correlate the staining data with functional assays, we also established bulk cultures from the same preparation of cells used in the staining experiments and assayed them for peptide-dependent cytotoxicity.

The highest frequency of T cells specific for either the Gag or Pol epitopes was found in patient 065, where 0.77% of the CD8+ small lymphocytes bound the A2-Pol tetramer, whereas a smaller number of cells (0.28%) were stained by the A2-Gag tetramer (Fig. 2A). In the samples from three additional patients, distinct peaks in the staining profiles were observed with the A2-Gag tetramer, whereas no staining was observed with the A2-Pol reagent (Fig. 2, B through D). Consistent with the staining results, the bulk culture cells from patient 065 killed target cells pulsed with the Pol peptide, whereas the bulk cultures from patients 868 (Fig. 2F) and 077 (Fig. 2G) exhibited the predicted killing activity toward Gag- but not Pol-loaded targets. The bulk cultures from the fourth patient killed neither Gag- nor Pol-loaded targets (Fig. 2H). In two cases (Fig. 2, A and D), the staining data predicts Gag-specific killing, although none was observed (Fig. 2, E and H). Although the percentage of A2-Gag+ cells in patient 065 (Fig. 2A) is significant, a distinct peak in the A2-Gag staining profile was absent, so that the 0.28% of A2-Gag+ CD8+ cells may represent an overestimate. Alterna-

Fig. 2. Correlation of antigen-specific staining in three of four patients with peptide-specific killing activity in CTL bulk cultures. Peripheral blood mononuclear cells from four healthy HIV-infected donors were separated as described (5). The CD4 counts for each patient at the time of analysis were as follows: patient 065, 410; patient 868, 330; patient 077, 270; and patient 605, 510. Two million peripheral blood cells from each patient were stained with anti-CD8a-CyChrome and phycoerythrin-labeled HLA-A2-Gag (solid line) or HLA-A2-Pol (dotted line) tetramers as indicated (A through D). Software gates were set to display only CD8+ small lymphocytes. The percentages of CD8+ cells that were positive for either A2-Pol or A2-Gag within gates as displayed are included in each panel. The reproducibility of the A2-Pol− and A2-Gag+ populations in patient 065 (A) was tested through analysis of five separate stains with each reagent; the standard deviations are reported. Bulk cultures were assayed for CTL activity (E through H) (19) on days 14 through 16 at an effector:target ratio of 50:1. Black bars, lysis of Gag-loaded targets; white bars, lysis of Pol-loaded targets.

Fig. 3. Analysis of surface phenotype of HLA-A2-Pol+ cells from patient 065. Peripheral blood cells were stained as described (18). Approximately 200,000 CD8+ small lymphocytes were analyzed for antigen specificity by using the A2-Pol tetramer and the expression of the following surface markers with fluorescein isothiocyanate-labeled antibodies as indicated: CD45RO (Dako, clone UCHL-1), CD38 (Coulter, MCA1019F), CD62L (Becton Dickinson, Leu-8, clone SK11), HLA-DR (Becton Dickinson, clone L243), and CD57 (Becton Dickinson, clone HNK-1). Contour plots were generated with CELLQuest software (Becton Dickinson) by using the 75% log density contour option to demonstrate small populations that stain positive for HLA-A2-Pol.

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tively, the A2-Gag$^+$ cells found in patients 065 and 606 (Fig. 2, A and D) may have failed to proliferate or develop effector function in bulk culture, possibly because of weaker proliferative capacity relative to other cells.

The frequency of antigen-specific T cells in patient blood that stain with our reagents is quite high but is consistent with previous estimates for anti-HIV CTL populations. LDA of anti-HIV CTL responses has suggested precursor frequencies in the range of 0.0001 to 0.003 (4, 21), but even these unusually high frequencies are thought to be an underestimate because an analysis of clones present in TCR cDNA libraries suggests frequencies as high as 1:100 (4, 5). This later estimate is supported by the more direct staining data we present.

The phenotypic state of a T cell is dependent on its contact with an antigen and can be analyzed with a variety of cell-surface markers (22, 23). Powerful techniques for phenotypic analysis of antigen-specific T cells use trace populations of transgenic T cells that can be labeled with clonotypic antibodies (23, 24); alternatively, cells from nontransgenic subjects can first be sorted according to phenotype, followed by assay for antigen specificity by LDA techniques (25). The MHC tetramers provide a more general, rapid, and direct method for analysis of the phenotypic state of antigen-specific T cells. To demonstrate this, we analyzed the phenotype of the A2-Pol–specific cells we found in the peripheral blood of patient 065 (Fig. 3). The CD8$^+$A2-Pol$^+$ population is composed almost exclusively of cells that are CD45RO$^+$ and CD62L$^-$, both indicating the memory and effector phenotypes expected for cells with a history of contact with antigen. The A2-Pol$^+$ cells are predominantly negative for the activation markers HLA-DR (26) and CD38 (27), which suggests a memory rather than effector phenotype; the A2-Pol$^+$ cells are also negative for CD57, a marker of unknown function found on elevated percentages of CD8$^+$ cells in HIV-infected patients (22). A similar bias toward a memory-cell phenotype was observed in the HIV antigen-specific cells of two other patients (28), suggesting that this may be a general feature of asymptomatic individuals.

The methodology we introduce here provides a powerful and general tool for the study of the development and phenotype of antigen-specific T cells. It does not require in vitro assays such as LDA to determine and quantify peptide-specific responses. Other approaches to the study of antigen-specific T cells in vivo require either the use of transgenic cells (23, 24) or that the TCR repertoire of the responding cells be unusually restricted and that antibodies to V$_\alpha$ and V$_\beta$ domains are available (1, 8). Staining T cells with a tetrameric peptide-MHC complex as described here solves many of the problems inherent in these techniques. The methodology is also generally applicable because it can be adapted for any T cell ligand by the engineering and expression of peptide-MHC complexes followed by tetramer synthesis.

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12. DNA coding for a Gly5er linker and a BSP (10) was fused to the 3' end of the soluble domain of the HLA-A2 heavy chain by polymerase chain reaction (PCR) with the 5' primer CAGAGGATGTATGGCTGC and the 3' primer GGGCAAGCTTTTAACGATGATTGCAGACC-ATTTTCTGTGCATCCAGAATATGATGCAGGGATC-CGGTGAGGGGCTTGGGCAA, by using an HLA-A2 expression plasmid as the template (11). The 5' primer lies upstream of a unique Sac I site in the HLA-A2 gene. The PCR product was digested with Sac I and Hind III and subcloned back into the expression plasmid, and the expressed protein was folded in vitro with β₂M in the presence of specific peptide ligands and initially purified as described (11). The A2-peptide complexes were further purified on a MonoQ column (Pharmacia) in 20 mM tris (pH 8.0), with a gradient of NaCl from 0 to 0.5 M. The purified proteins were stored in phosphate-buffered saline (PBS) plus a cocktail of protease inhibitors: pepstatin, 0.7 μg/ml; leupeptin, 1 μg/ml; phenylmethylsulfonyl fluoride, 2 μM; and EDTA, 1 mM. Peptides were ILKEPVHGV (Pol), SLYNTVATL (Gag), or GILGFVFTL (MP) (29) and were synthesized in the Stanford Protein and Nucleic Acid Facility. Purified HLA-A2-BSP-peptide complexes were enzymatically biotinylated by incubation with purified BirA for 12 hours at 25°C with components as follows: HLA-A2-BSP, 5 μM; BirA, 0.1 μM; tris (pH 7.4), 50 mM; sodium chloride, 150 mM; biotin, 1 mM; adenosine triphosphate, 5 mM; and MgCl₂, 5 mM. BirA was produced from the overexpression plasmid pJS169 in *Escherichia coli* BL21 and purified on Blue Sepharose, followed by ion exchange on S Sepharose Fast Flow (P. Schatz and R. Armstrong, personal communication). Biotinylation levels were usually between 70 and 100%. HLA-A2 tetramers were prepared by mixing the biotinylated protein with phycoerythrin-labeled UltraAvidin (Leinco) at a molar ratio of 4:1. Tetramers were purified by gel filtration on a Superdex S-200 column (Pharmacia).
13. Our attempts to produce effective avidin-based staining reagents from randomly biotinylated MHC molecules were unsuccessful, though this approach has succeeded in other systems [C. R. Parish, M. A. Reeny, M. H. Knoppers, J. C. Waldron, H. S. Warren, *J. Immunol.* 150, 4839 (1993)].
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17. Viral peptide-specific CTLs were generated as described (20, 30). Briefly, peripheral blood mononuclear cells were separated from whole blood and restimulated with autologous phytohemagglutinin lymphoblasts. Cells were cultured in RPMI 1640 (Gibco) with 10% fetal calf serum (R10) and antibiotics for 1 week, and subsequently with R10 and 10% Lymphocult T (Biotest) added. Viral peptide-specific lines were generated and maintained from the bulk culture by use of peptide-pulsed irradiated autologous B-lymphoblastoid cells weekly as feeders. The HLA-A2-Pol-specific clone 20 was cloned by LDA techniques.
18. For staining, approximately 200,000 CTLs were incubated at 4°C for 1 hour with saturating concentrations of CyChrome-conjugated antibody to CD8 (clone RPA-T8, Pharmingen) and phycoerythrin-labeled HLA-A2-peptide tetramers at a concentration of HLA-A2 of approximately 0.5 mg/ml total in PBS plus 2% fetal calf serum plus 5 mM sodium azide. After washing, the cells were fixed in PBS plus 2% formaldehyde. We then analyzed the cells on a FAC-Scan (BDIS), using CELLQuest software.
19. Peptide-specific lines were assayed 4 to 5 days after restimulation. Standard 4-hour chromium-51 release assays were performed with autologous chromium-51-labeled B-lymphoblastoid cells as targets. Background chromium release was less than 20%. Percent lysis was calculated from the formula 100 × (E − M)/(T − M), where E is the experimental release, M is the release in the presence of R10 media alone, and T the release in the presence of 5% Triton X-100 detergent.
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Live Salmonella as vaccines and carriers of foreign antigenic determinants

Steven N. Chatfield, Richard A. Strugnell and Gordon Dougan

Salmonella species can be rationally attenuated by introducing non-reverting defined mutations into the genome to produce live vaccine strains. Several genes have been identified which when mutated, will attenuate Salmonellae. *In particular, salmonella strains harbouring mutations in genes involved in the pre-chorismate biosynthetic pathway make excellent oral vaccines evoking strong humoral, local and cellular immune responses in the host. Because of the spectrum of immune responses induced by live vaccine strains they have the potential to be used for delivery of heterologous antigens to the mammalian immune system. A number of antigens from other bacteria, viruses and parasites have been expressed in live salmonella vaccine strains. Such hybrid strains have the potential to be used as multivalent vaccines against a number of infectious diseases.*

Salmonella infections remain a serious world health problem. These pathogens form a closely related group of species which are capable of causing a variety of diseases in mammals ranging from a localized gastro-enteritis to disseminated infections involving organs of the mononuclear phagocyte system (MPS), namely the spleen, liver, lymph nodes and bone marrow. In man, *Salmonella typhi*, the cause of typhoid is the most dramatic example of an invasive salmonella although other salmonella species can cause systemic infections. Work on the development of new typhoid vaccines has shown that live attenuated salmonella strains can elicit protective immunity and induce secretory, humoral and cellular anti-salmonella responses in the host after oral administration. Thus live salmonella vaccines are potentially potent oral immunogens. Since there is no realistic small animal model for studying *S. typhi* infections much of the work carried out on developing novel typhoid vaccines has utilized mouse virulent strains of salmonella such as *S. typhimurium*, *S. dublin* and *S. enteriditis*. These strains cause invasive infections in susceptible mice which resemble typhoid in humans[1-3]. The use of the murine typhoid model has led to the construction of a number of rationally attenuated salmonella vaccine strains that have the potential to protect against typhoid and other salmonella infections

and also to be used for the oral delivery of heterologous antigens to the immune system. The use of such strains will be described in this review.

Methods of attenuating virulent salmonella strains

Attenuated salmonella strains could provide a route for the development of a range of practical oral vaccines. Any salmonella strain considered for use as a carrier should be attenuated in a genetically defined manner and be well characterized *in vivo*. Early attempts to attenuate salmonella involved the use of mutants of *S. typhi* dependent on streptomycin for growth *in vitro*[4,5]. Further attempts to produce attenuated salmonella mutants used chemical mutagenesis[6]. Although useful information was obtained, and a practical oral typhoid vaccine, Ty21a[7], was approved for use in humans, this approach has been superceded by more defined genetic techniques. A number of individual genes in which mutations give rise to attenuated strains have been identified and these are listed in *Table 1*.

The most exhaustive studies on genetically defined attenuated variants have been carried out on auxotrophic mutants. Following up observations made in the 1950s[8,9] Hoiseth and Stocker[10] described the construction of *S. typhimurium* strains harbouring tranposon insertions in the *aroA* gene. Salmonella *aroA* strains were found to be highly attenuated in mice and proved to be excellent single dose oral vaccines against salmonellosis. Subsequently *aroA* mutants of salmonella have been tested extensively in mice[11-13], calves[13,14] and sheep[15] and were found to be attenuated and able to induce protective immunity. The *aroA* gene located at 19 min on the *S. typhimurium* genetic map[16] encodes 5-enolpyruvylshik-

Table 1 Individual genes in which mutations give rise to attenuation in Salmonella sp.

Gene	Enzyme/function	Reference
aroA,C,D	Aromatic compound biosynthesis Pre-chorismate pathway	10, 12–15. 17, 18, 21, 22
purA,B,E,H	Purine biosynthesis	12, 20
galE	UDP glucose 4-epimerase	47
cya	Adenylate cyclase	23
crp	cAMP receptor protein	23
ompR	Outer membrane protein. modulates porin expression	24
phoP	Non-specific acid phosphatase	25

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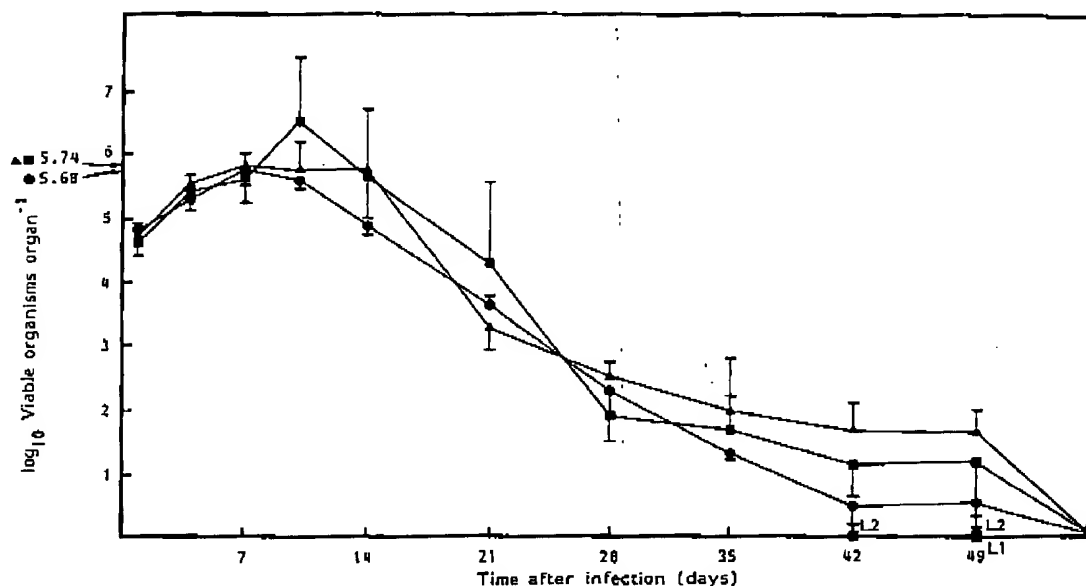
Figure 1 Colonization of livers and spleens of Balb/c mice after i.v. infection with *S. typhimurium*: ●, $\log_{10}$ 5.68 aroA; ▲, $\log_{10}$ 5.74 aroC; ■, $\log_{10}$ 5.68 aroA aroC. Each point represents the geometric mean ± two standard errors for four mice. Counts in individual mice when one or more of the group of four organs had no detectable salmonellae are indicated (L1, L2)

imate-3-phosphate synthetase, the penultimate enzyme of the early common prechorismate pathway leading to the synthesis of chorismate and then to aromatic amino acids, *para*-aminobenzoic acid, and 2.4-dihydroxybenzoate. *Aro* mutants are dependent on these compounds for growth *in vitro*. Mammals do not possess this pathway and it is likely that the availability of some of these compounds *in vivo* is limited.

Following on from the work with mouse virulent salmonella, auxotrophic mutants of *S. typhi* were constructed[17,18]. To reduce the possibility of reversion to virulence it was thought wise to introduce two independent, stable attenuating mutations that map at widely separate locations on the chromosome. To this end Levine and colleagues[19] constructed strains of *S. typhi* harbouring *aroA purA* mutations. *PurA* is a gene involved in purine biosynthesis and it had previously been shown that purine dependent salmonella strains were attenuated[12,20]. The *S. typhi aroA purA* strains were fed to human volunteers and were found to be attenuated but poorly immunogenic[19].

Extensive studies with single and double auxotrophic mutants constructed by introducing different stable *aro* and *pur* deletion mutations into mouse virulent *S. typhimurium* showed that strains harbouring single and different combinations of auxotrophic mutations differed substantially in their immunogenicity[13,21,22]. *PurA* mutants were found to be highly attenuated, more so than *aroA* mutants. This was reflected by the patterns of *in vivo* persistence after intravenous administration in the MPS of mice, *purA* mutants persisting for longer but not reaching such high levels in livers and spleens as *aroA* mutants. Combined *aroA purA* mutants had a different pattern of persistence distinct from that of either single mutant. In terms of vaccine potential *aroA* mutants were excellent live vaccines while *purA* and *aroA purA* were poor vaccines in terms of protection against virulent challenge. To explain these differences further studies were carried out to determine humoral and cellular

immune responses to these mutants. It was determined that both *aroA* and *purA* vaccine strains elicited strong humoral antibody responses although the response to *aroA* strains was significantly higher and *aroA* strains were found to be much more effective macrophage activators than *purA* strains (our unpublished observations).

The main conclusions drawn from these extensive animal studies was that strains harbouring single and double combinations of mutations in the pre-chorismate biosynthetic pathway such as *aroA*, *aroD* or *aroC* behaved in a similar manner in terms of attenuation and *in vivo* persistence and all were excellent single dose oral and parenteral vaccines. *Figure 1* demonstrates the similarity of these strains by comparing the *in vivo* persistence in livers of Balb/c mice of *aroA*, *aroC* and *aroA aroC S. typhimurium* SL1344 after intravenous administration. All strains exhibited a very similar pattern of persistence with no salmonellae detectable for any of the strains by day 56. In contrast *purA* mutations were too attenuated to provide protection against salmonellosis in a single oral dose although they were still able to stimulate local secretory immune responses. However, similar *S. typhi* derivatives have not been fully tested in humans.

More limited studies have been carried out with salmonella strains harbouring other attenuating lesions. *S. typhimurium* strains harbouring deletions in *cya* (adenylate cyclase) and *crp* (cyclase receptor protein) have been constructed[23] and were found to be attenuated and effective as oral vaccines. It has recently been shown that *S. typhimurium* strains harbouring stable mutations in *ompR*, a positive regulator of porin expression, are attenuated following oral or parenteral administration to mice[24]. These strains were also effective oral vaccines[24]. salmonella strains harbouring mutations in another regulatory gene, *phoP*, have recently been shown to be attenuated[25]. Apparently mutations in *phoP* impaired the ability of salmonella strains to survive intracellularly inside macrophages[25].

Mutagenesis methods are now available to target mutations into secreted bacterial proteins with the idea that most proteins which play a role in bacterial virulence are located at, or pass through, the surface of the bacteria where they can readily interact with the host. One such system uses TnphoA, a hybrid Tn5 transposon[26]. This approach has been used to isolate several orally attenuated *S. typhimurium*[27] and *S. choleraesuis* mutants[28].

Attenuated salmonella strains as carriers of heterologous antigens

It is now clear that there are available a series of rationally attenuated salmonella vaccine strains capable of eliciting varying degrees of immune responses after oral administration. These properties make salmonella an attractive carrier of potential protective heterologous antigens to the mammalian immune system. Salmonella can survive and grow within macrophages and other professional phagocytic cells[29] and this property may have important consequences for the abilities of salmonella vaccines to present cloned antigens.

S. typhimurium is closely related to *Escherichia coli*, the organisms sharing much of the same genetic information. Much of what is known about bacterial genetics stems from work with these two organisms and thus systems for conjugation, transformation and transduction are well developed in salmonella. DNA sequences which direct the expression of antigens in *E. coli* will normally express in salmonella making it possible to clone and characterize a potentially useful antigen in *E. coli*, before transferring into salmonella vaccine strains for evaluation. The major problem encountered whilst attempting to express foreign genes in salmonella vaccine strains is plasmid instability and this has been discussed extensively elsewhere[30,31].

Much of the early heterologous antigen carrier work utilized the *S. typhi* human oral typhoid vaccine strain Ty21a[6]. A *Salmonella–Shigella* hybrid was constructed by moving the virulence-associated plasmid of *S. sonnei* that encodes genes for the biosynthesis of the *Shigella* form 1 antigens into Ty21a. Hybrid strains expressed the antigen on the surface as well as 09 and 012 somatic antigens of *S. typhi*[32]. This strain was used to vaccinate mice and induced immune responses to both *S. sonnei* and *S. typhi* antigens. Moreover, this bivalent vaccine has been shown to protect human volunteers against challenge with *S. sonnei*[33].

Early work with salmonella vaccine strains harbouring defined mutations utilized the heat-labile toxin B subunit (LT-B) of enterotoxigenic *E. coli* (ETEC). LT-B heat-labile toxin is a two subunit toxin secreted by many ETEC isolates[34,35]. The B subunit is highly immunogenic and non-toxic and could be a component of an enterotoxigenic *E. coli* (ETEC) or *Vibrio cholerae* vaccine. Strains of *S. typhimurium* and *S. enteritidis* have been constructed which express LT-B[36,37]. Mice fed these strains developed high titres of gut-associated IgA against both salmonella and LT-B. Serum antibody also neutralized the activity of labile toxin against susceptible tissue culture cells. LT-B has also been introduced into Ty21a[38]. Such a hybrid strain might form the basis of a triple vaccine against typhoid, cholera and enterotoxigenic *E. coli*-related diarrhoeal diseases.

The antigens discussed so far are all from closely related organisms. It is now possible, with modern genetic

techniques, to introduce antigens from other prokaryotes and higher organisms such as parasites into salmonella. Poirier *et al.*[39] have recently reported the transformation of a *S. typhimurium aroA* strain with a plasmid encoding the M5 protein of *Streptococcus pyogenes*. The M protein is a major virulence factor of *S. pyogenes*. When mice were infected orally with this hybrid strain they developed serum and mucosal antibody responses against the type M5 protein. Furthermore the mice were completely protected against both intranasal and intraperitoneal challenge with M5 producing streptococci.

With regard to antigens from higher organisms there are, to date, few examples. Taylor *et al.*[40] have expressed a cloned antigen from *Schistosoma mansoni* in galE and aroA *S. typhimurium* strains but failed to elicit an immune response against the antigen in mice. More recently Sadoff *et al.*[41] have introduced the circumsporozoite protein of a rodent malaria parasite, *Plasmodium berghei*, into an avirulent salmonella strain which was used to orally vaccinate mice. Protection against sporozoite challenge was demonstrated five weeks after vaccination in the absence of detectable antibody, indicating the induction of a specific anti-plasmodium cellular response.

Some investigators believe that it is advantageous to present heterologous antigens on the surface of the delivering organism. Several secretion systems have been developed for expressing foreign antigenic determinants at the bacterial cell surface. For example peptides can be expressed at sites within the *E. coli* LamB and PhoE proteins and the sequences can be detected at the cell surface. IamB[42] and phoE[43] genes encode outer membrane proteins. Newton *et al.*[44] recently described the use of flagella of salmonella for presenting foreign antigens. They inserted a synthetic oligonucleotide encoding an epitope of cholera toxin subunit B into flagella of an aroA *S. dublin*. When given to mice this hybrid strain evoked a humoral antibody response to cholera toxin.

It has also been demonstrated that β-galactosidase, an intracellular protein from *E. coli*, elicits a good humoral and cellular immune response in mice when delivered using attenuated salmonella[45]. This infers that it may not be necessary to express the heterologous antigen on the bacterial cell surface to generate a strong immune response. Indeed we have recently found that salmonella expressing the influenzae virus nucleoprotein as an intracellular antigen[46] can stimulate humoral and cytotoxic T-cell responses to the nucleoprotein in mice (J. Tite, personal communication).

Conclusions

Techniques are now available for introducing defined genetic lesions into virulent pathogens such as *S. typhi*. Some of these rationally attenuated live vaccines provide good protection against homologous challenge and can act as carriers of foreign genes. Such bivalent or multivalent oral vaccines may offer important advances in immunization against infectious diseases.

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Biological and immunogenic properties of a canarypox–rabies recombinant, ALVAC-RG (vCP65) in non-avian species

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A canarypox-based (ALVAC) recombinant expressing the rabies G glycoprotein has been utilized to assess in vitro and in vivo biological properties of the canarypox virus vector system. In vitro studies have shown that no replication of the virus can be detected on six human-derived cell lines, nor can the virus be readily adapted to replicate on non-avian cells. Expression of the rabies G can be detected on all cell lines analyzed in the absence of productive viral replication. Analysis of viral-specific DNA accumulation indicated that the block in the replication cycle in the human cell lines analyzed occurred prior to DNA replication. The exact nature of the block, however, remains unknown. The concept of using a non-replicating immunization vehicle has been demonstrated through extensive in vivo studies in a range of species including non-human primates and humans. The results of such in vivo studies have exemplified the safety and immunogenicity of the ALVAC vaccine vector.

The development of naturally host-restricted avipox virus vectors capable of expressing extrinsic immunogens and inducing a protective immune response against lethal viral challenge in mammalian species has been described[1–3]. Fowlpox virus (FPV) and canarypox virus (CPV) are members of the avipox virus genus of the Orthopoxvirus family. Productive replication of avipox viruses is restricted to avian species[6]. Both FPV and CPV-rabies recombinants[1,3] express the rabies glycoprotein in tissue culture cells of non-avian origin without apparent replication of the vector virus. Inoculation of these recombinants into a range of non-avian species including mice, cats, and dogs demonstrated that the level of expression of the foreign gene product was sufficient to induce rabies-specific serum neutralizing antibodies and to protect against a lethal rabies virus challenge.

Potency tests in mice indicated that a CPV vector expressing the rabies glycoprotein was 100-fold more efficacious than an FPV-based vector and that the

protective efficacy of a host-restricted CPV-rabies vector was similar to that of a replication-competent vaccinia virus vector containing the rabies G gene in the thymidine kinase locus[2]. Further, both replication competent VV-measles recombinants and a host-restricted CPV-measles recombinant induced similar levels of measles virus neutralizing antibody and protection against experimental canine distemper virus challenge in dogs[5].

Additional studies have shown that the utility of avipox vectors as immunizing agents in non-avian species is not limited to the rabies glycoprotein or measles virus immunogens. Vaccination of cats with an ALVAC-based recombinant expressing the feline leukemia virus (FeLV) A subtype Env and Gag proteins protects against the development of persistent viremia following FeLV challenge exposure[7]. ALVAC recombinants expressing immunogens from Japanese encephalitis virus (JEV) have also been shown to protect mice against a lethal JEV challenge[8]. Safety and immunogenicity studies in horses utilizing an ALVAC recombinant expressing the hemagglutinin glycoproteins from the A1 and A2 serotypes of equine influenza virus demonstrated the induction of type specific hemagglutination-inhibiting antibodies and protection against an A2 epizootic[4]. An ALVAC-based recombinant expressing the HIV-1 envelope glycoprotein has recently been shown to induce HIV-specific antibody and cytotoxic T-lymphocyte responses in mice[9]. These examples of ALVAC recombinants expressing immunogens from a variety of viral pathogens indicate the general utility of ALVAC-based

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recombinant viruses as immunization vehicles in a variety of mammalian species.

Avipox virus-based vectors theoretically provide significant safety advantages in light of their inability to productively replicate in non-avian species. Such a vaccine vector should not allow dissemination within the vaccinated individual, contact transmission to non-vaccinated individuals, or general contamination of the environment. The concept of utilizing a replication-restricted vaccine vector, ALVAC-RG, in humans has recently been assessed with promising results. The experimental vaccine was well tolerated and induced protective levels of rabies-neutralizing antibodies[10,11].

These studies in target species have provided a database which has indicated that ALVAC-based recombinants may have significant advantages as vector-based vaccines. It therefore was critical to rigorously evaluate the safety characteristics of ALVAC-based recombinants in laboratory animals and to establish the innocuity and immunogenicity of the vector in both non-human primates and target species. This report describes *in vitro* and *in vivo* studies designed to explore the basis of host restriction, safety, and immunizing potential of the ALVAC-based vector.

MATERIALS AND METHODS

Cells and viruses

Viral amplifications and plaque titrations were performed on primary chicken embryo fibroblast (CEF) cells from 10 to 11 day embryos of SPF origin. Titrations were also performed as a microtiter assay on the quail QT35 cell line[12] and titers determined by the method of Karber[13].

The origin of other cells used in this study is as follows: (1) VERO cells (ATCC No. CCL81) are a line derived from African Green Monkey kidney; (2) MRC-5 (ATCC No. CCL171) are of human embryonic lung origin; (3) HNK are human neonatal kidney cells subcultured for less than five passages (Whittaker BioProducts, Inc., Walkersville, MD (Cat. No. 70-151)); (4) HEL 299 are human embryonic lung cells (ATCC No. CCL137); (5) WISH are of human amnion origin (ATCC No. CCL25); (6) DETROIT 532 are of human foreskin (Down's Syndrome) origin (ATCC No. CCL54); (7) JT-1 is a human lymphoblastoid cell line transformed with Epstein-Barr virus as described in Rickinson *et al.*[14]

The canarypox virus strain from which ALVAC was derived was isolated from a pox lesion on an infected canary. The virus was first isolated at the Rentschler Bakteriologishes Institut, Lauphein, Wurtemberg, Germany, where it was attenuated by 200 serial passages in CEFs. This attenuated strain (Kanapox) obtained from Rhone Merieux is licensed as a vaccine for canaries in France. At Virogenetics, the virus was subjected to four successive rounds of plaque purification under agarose. One plaque isolate, designated ALVAC, was selected for amplification and used in these studies.

Development of recombinant ALVAC-RG (vCP65)

The canarypox rabies recombinant was derived by methods previously described[1,15,16]. A unique insertion locus was defined at a *Bgl*II site within an 880 bp *Pvu*II

fragment of CPV genomic DNA. The DNA sequence of this fragment was determined and the open reading frame (ORF) designated as C5 defined. Deletion of the entire C5 ORF was made by standard molecular biological procedures[17,18] without interruption of neighboring ORFs. The C5 ORF was replaced by *Hind*III, *Sma*I and *Eco*R1 insertion sites followed by translation termination codons and early vaccinia virus transcription termination signals[19].

The ERA strain rabies glycoprotein cDNA[20,21] linked to the early/late vaccinia virus H6 promoter[1,16,22] was inserted at the *Sma*I site. The resulting plasmid, pRW838, was transfected into ALVAC-infected primary CEF cells using the calcium phosphate precipitation method[15]. Plaques were selected on the basis of DNA hybridization to a rabies G-specific radiolabelled probe and subjected to sequential rounds of plaque purification. A representative plaque was then amplified and designated ALVAC-RG with the laboratory designation of vCP65.

Inoculation of non-avian cells with ALVAC-RG

A variety of human cell substrates, MRC-5, HNK, HEL, DETROIT-532, WISH and JT-1, were inoculated with ALVAC-RG and analyzed for expression of the rabies G gene, and accumulation of viral-specific DNA. Primary CEF cells were included as a permissive substrate.

Viral DNA accumulation

Sixty millimetre dishes containing two million cells of each cell type under test were inoculated with ALVAC at a multiplicity of infection (MOI) of 5 p.f.u. per cell. After an adsorption period of 1 h at 37°C, the inoculum was removed, the monolayer washed twice to remove unadsorbed virus and the infected monolayer refed with 5 ml of Eagle's Minimal Essential Medium (EMEM) + 2% Newborn Calf Serum (NCS). Cells from one dish were harvested at t_0 and the remaining dishes were incubated, in the presence or absence of 40 μg ml^{-1} of cytosine arabinoside (AraC; Sigma No. C6654), at 37°C for 72 h. Cells were collected and resuspended in 0.5 ml phosphate buffered saline (PBS) containing 40 mM EDTA and incubated for 5 min at 37°C. An equal volume of 1.5% agarose containing 120 mM EDTA, prewarmed to 42°C, was gently mixed with the cell suspension and transferred to an agarose plug mold. After solidification, the agarose plugs were removed and incubated for 12–16 h at 50°C in a volume of lysis buffer (1% sarkosyl, 100 μg ml^{-1} proteinase K, 10 mM Tris HCl pH 7.5, 200 mM EDTA) sufficient to cover the plug. The lysis buffer was then replaced with 5 ml 0.5×TBE (44.5 mM Tris borate, 44.5 mM boric acid, 0.5 mM EDTA) and equilibrated at 4°C for 6 h with 3 changes of 0.5×TBE buffer. The viral DNA within the plug was fractionated from cellular nucleic acid using a BIO-RAD CHEF-DR II pulse field electrophoresis system (180 V/20 h/15°C) in 0.5×TBE with a ramp time of 50–90 s, using lambda DNA as molecular weight standards. The viral DNA band was first visualized by staining with EtBr, then transferred to a nitrocellulose membrane and probed with a radiolabelled probe prepared from purified canarypox genomic DNA.

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Analysis of expression of rabies G gene

Immunoprecipitation analysis was performed from a radiolabelled lysate of each cell line infected with ALVAC or ALVAC-RG (vCP65) as described in Tartaglia et al.[18] using a rabies glycoprotein-specific monoclonal antibody designated 24-3F10.

Time course study

MRC-5 and CEF monolayers were inoculated with 10 pfu/cell of ALVAC or ALVAC-RG (vCP65) at 37°C for 60 min. The inoculum was removed, the monolayer washed twice, and the medium replaced. At 1, 3, 5, 9, 13, 17, and 25 h post infection, the culture was labelled for 1 h by the addition of methionine-free medium containing 25 μCi ml^{-1} of ^{35}S-methionine (DuPont NEN; 1140 Ci mmol^{-1}). Infected cells were scraped from the culture dishes, collected by centrifugation, washed twice with PBS and lysed by the addition of 2 ml of Buffer A (18). Infected cell lysates were analyzed for expression of the rabies G gene by immunoprecipitation as described in Tartaglia et al.[18]

Safety studies in laboratory animals

Groups of rabbits (New Zealand white ESD), guinea pigs (Dunkin-Hartley, Libeau) and mice (IFFA Credo, Les Oncins, France) were inoculated with ALVAC-RG by a variety of routes as shown in *Table 1*. Animals were inspected daily for signs of reactogenicity and at the termination of the test at 14–21 days, animals were euthanized and tissue at the site of inoculation examined. To monitor neurovirulence, nine male OF$_1$ mice were anesthetized and injected by the i.c. route with ALVAC-RG (vCP65). Three mice were inoculated with an uninfected cell extract. Three inoculated and one control mice were sacrificed on days 1, 3 and 6 post-inoculation. Brains were fixed in situ by immersing the opened skull in a solution of buffered formalin. After processing, 5 sections were made and stained with gallocyanin/phloxine. The sections involved the following levels: A: corpus striatum, B: infundibulum, C: pedunculi cerebri, D: pons and E: cerebellum.

Comparison of virulence of Kanapox virus (Rentschler strain of CPV) and ALVAC-RG in canary birds

Canary birds certified to have not been immunized with canarypox virus were obtained from PIC Grains (Vignouse sur Barangeon, France). Birds were inoculated with 5.0 or 7.0 log$_{10}$ p.f.u. of either Kanapox (Rentschler strain of CPV) or ALVAC-RG (vCP65) by

Table 1 Safety studies in laboratory animals: schedule of inoculation by different routes

Species	Virus	Dose	Route	Volume	Sites
Rabbit	ALVAC	5.7*	i.c.	0.1 ml	1
	ALVAC-RG	5.7	i.c.	0.1 ml	1
	ALVAC-RG	6.3	i.d.	0.2 ml	5
	ALVAC-RG	8.0	s.c.	9.0 ml	1
Guinea pig	ALVAC-RG	6.0	i.d.	0.1 ml	5
	ALVAC-RG	7.3	s.c.	2.0 ml	1
Mice	ALVAC-RG	5.7	i.d.	0.05 ml	5
	ALVAC-RG	6.7	s.c.	0.5 ml	1
	ALVAC-RG	6.0	i.c.	0.05 ml	1

*Inoculum dose expressed as log$_{10}$TCID$_{50}$

smearing 50 μl of a 1:1 mixture of virus suspension and glycerin on a 0.5 cm^2 area from which the feathers had been removed on the back of each bird. Birds were monitored on a daily basis for one month postinoculation with weighing at 2–3 day intervals.

Inoculation of ALVAC-RG into the skin of canary birds and mice

Female OF$_1$ mice were injected by the i.d. route in each ear pinna with 5.0 log$_{10}$ TCID$_{50}$ of ALVAC-RG (vCP65) in 20 μl. Canary birds received an equivalent dose mixed with glycerin and smeared on a 1.0 cm^2 area of skin on the back from which feathers had been removed. At time intervals, animals were sacrificed and the skin surrounding the site of inoculation was dissected, homogenized in medium 199/Ham F10 plus 2% FCS, and stored at −70°C. Mouse specimens consisted of the entire skin covering the dorsal face of the ear pinna. Homogenates were thawed, sonicated, centrifuged, diluted 1:100 to avoid toxicity, and titrated in serial dilutions in QT35 cells.

Immunogenicity and safety studies in primate species

Three species of non-human primate, rhesus macaques, chimpanzees and squirrel monkeys (*Saimiri sciureus*) were inoculated with ALVAC-RG as shown in *Table 2*. The study in squirrel monkeys also addressed the questions of the ability to re-isolate virus after inoculation by a variety of routes and the immune response to ALVAC-RG in the face of pre-existing ALVAC immunity. In this study, three groups of four squirrel monkeys were inoculated with one of three viruses: (a) ALVAC, the parental canarypox virus; (b) ALVAC-RG (vCP65); or (c) ALVAC-FL (vCP37), a canarypox recombinant expressing the envelope glycoprotein of FeLV (Tartaglia et al., unpublished data). Inoculations were performed under ketamine anesthesia. Each animal received at the same time: (1) 20 μl instilled on the surface of the right eye without scarification; (2) 100 μl as several droplets in the mouth; (3) 100 μl in each of two i.d. injection sites in the shaven skin of the external face of the right arm; and (4) 100 μl in the anterior muscle of the right thigh. In each group, two animals received 5.0 log$_{10}$ p.f.u. and two animals received 7.0 log$_{10}$ p.f.u. of the appropriate virus. Virus isolation was attempted from the site of inoculation for 11 days post-inoculation and all monkeys were monitored for adverse reactions. Six months after the initial inoculation, selected animals from each group plus one canarypox naive animal were inoculated with ALVAC-RG (vCP65) as described in *Table 2*. All animals were monitored for adverse reactions to vaccination and sera analyzed for the presence of anti-rabies antibodies[24].

RESULTS

Derivation of ALVAC-RG (vCP65)

The strategy used to develop FPV[1,16,23] and CPV[2,3] recombinants involved insertion of the foreign gene at a unique restriction site within an ORF previously defined as nonessential. No attempt was made to precisely delete the interrupted ORF. In the generation of ALVAC-RG (vCP65), an insertion plasmid containing the H6/rabies G expression cassette was constructed

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Table 2 Schedule of inoculation of primate species with ALVAC-RG (vCP65)

Species	Designation	Dose[a]	Route	Previous inoculations	Booster dose. Interval
Rhesus macaque	177 and 186	7.7	s.c.	none	7.0, 100 days
	178	7.0	s.c.	none	none
	182	7.0	i.m.	none	none
	179	6.0	s.c.	none	none
	183	6.0	i.m.	none	none
	180	5.0	s.c.	none	none
	184	5.0	i.m.	none	none
Chimpanzee	431	7.0	i.m.	none	7.0, 84 days
	457	7.0	s.c.	none	7.0, 84 days
Squirrel monkey	37, 53	6.5	s.c.	5.0, ALVAC-RG	180 days
	38, 54	6.5	s.c.	7.0, ALVAC-RG	180 days
	22, 51	6.5	s.c.	5.0, ALVAC	180 days
	39, 55	6.5	s.c.	5.0, ALVAC-FL[b]	180 days
	57	6.5	s.c.	none	none

[a]Virus dose expressed as $\log_{10}$ p.f.u. per ml
[b]A canarypox recombinant expressing the envelope glycoprotein of FeLV (Tartaglia *et al.*, unpublished data)

such that the flanking arms of the plasmid directed replacement of the non-essential ORF with the foreign gene. Insertion of the foreign gene was accomplished without altering neighboring ORFs and the generation of novel ORFs was precluded by engineering translational stop codons in all appropriate reading frames. The derived recombinant, ALVAC-RG (vCP65), was confirmed to contain the rabies G expression cassette in the correct C5 locus by Southern blot analysis, PCR analysis, and nucleotide sequence analysis (data not presented). Further, expression analyses by immunofluorescence and immuno-precipitation using a rabies G glycoprotein-specific monoclonal antibody confirmed the expression of the 66 kDa rabies glycoprotein on the surface of ALVAC-RG infected cells.

In vitro studies

Analysis of expression of the rabies G gene in avian and human derived cells. Prior results have indicated that ALVAC and derived recombinants do not productively replicate in a range of non-avian cell lines, including those derived from monkey, mouse, cat and human[18] (unpublished results). Additionally, in a similar study to that described in Taylor *et al.* [1] attempts to adapt ALVAC and ALVAC-RG (vCP65) to grow in two non-avian cell lines (MRC-5 and VERO) have failed[25]. Blind passages of both ALVAC and ALVAC-RG were performed in VERO. MRC-5 and primary CEF monolayers for 8 or 10 sequential passages of 7 days duration. While a 100-fold increase in viral titer was apparent in CEF cells after each passage in the series, after one passage in mammalian cells, the viral titer was lower than the residual input titer and titers fell below the level of detectability after two passages[25].

In order to establish that in the absence of productive viral replication the rabies glycoprotein (G) was expressed in the human derived cell lines, immunoprecipitation experiments were performed. The results of a representative analysis are shown in *Figure 1*. No specific immunoprecipitation products were detected in lysates derived from uninfected cells (lanes a, d and g) or cells infected with the parental ALVAC virus (lanes b, e and h). Immunoprecipitation of a 66 kDa protein by the rabies-specific monoclonal antibody was apparent from lysates derived from ALVAC-RG

infected CEF, HNK and HEL cells (*Figure 1*, lanes c, f and i, respectively). This size is consistent with that described for SDS–PAGE of the rabies glycoprotein G[7].

In order to determine whether expression of the rabies G gene product would be maintained in human derived cells inoculated with ALVAC-RG (vCP65) in the absence of productive replication, a time course study was performed as described in Materials and Methods. Immunoprecipitation of the rabies G is shown in *Figures 2a* (CEF cells) and 2b (MRC-5 cells). Expression of the rabies G in both CEF and MRC-5 cells occurs as early as 1 h post-infection and continues undiminished under the control of the early/late H6 promoter throughout the labelling period of 24 h.

Analysis of viral specific DNA accumulation in human derived cells inoculated with ALVAC-RG (vCP65). In order to assess the temporal nature of the block in viral replication in human derived cells, the following experiment was performed. Permissive CEF cells and the six human derived cell lines were inoculated with ALVAC parental virus at an MOI of 5 pfu per cell in the presence or absence of AraC, an inhibitor of DNA replication, and the level of virus specific DNA accumulated at 72 h was assessed as described in Materials and Methods. *Figure 3* illustrates analysis of CEF, WISH and DETROIT 532 cells. In the permissive cell line, CEF (*Figure 3*; Panel B), no viral-specific DNA is seen in lane B1 (uninfected CEF cells), lane B2 (ALVAC-infected CEF cells at t_0) or lane B4 ALVAC infected CEF cells at 72 h post-infection in the presence of AraC). Viral specific DNA accumulation represented by a band at approximately 330 kbp is evident in ALVAC infected CEF cells incubated for 72 h in the absence of AraC (lane B3). No such accumulation is seen in the equivalent sample of ALVAC infected DETROIT-532 (lane A3) or WISH (lane C3) cells. Similar results were observed on analysis of ALVAC specific DNA accumulation in MRC-5, HEL, HNK and JT-1 infected cells (results not shown). Based on the conditions employed in these studies, the sensitivity of detection was determined as ≥ 125 genome equivalents. In further experiments, ^{3}H-thymidine incorporation into ALVAC-infected MRC-5 and CEF cells was monitored. These experiments indicated that while an increase in ^{3}H-thymidine incorporation occurred in CEF cells

following infection with ALVAC, in MRC-5 cells, ^{3}H-thymidine incorporation did not rise above basal levels (results not shown). The results indicate that under these conditions, no detectable ALVAC-specific DNA accumulation occurred in the human cell substrates and suggest that replication of ALVAC in these human cells is blocked prior to viral DNA synthesis.

In vivo safety studies with ALVAC-RG (vCP65)

Inoculation of laboratory animals. Previous experiments with CPV and FPV based recombinants in numerous species including mice, rabbits, rats, guinea pigs, cats, dogs, horses, cattle, and swine had demonstrated no adverse reactions upon inoculation by a variety of routes. It was important, however, to examine more stringently the safety profile of an ALVAC-based recombinant in mammalian species.

In a series of experiments described in Materials and Methods and *Table 1*, mice, guinea pigs and rabbits were inoculated with ALVAC-RG by a variety of routes

and reactivity monitored. The results indicated that no reactivity was evident in any species following inoculation by the subcutaneous route. Similarly, there was no evidence of neurovirulence apparent in mice or rabbits inoculated with approximately 6.0 log$_{10}$ TCID$_{50}$ of ALVAC-RG by the intracranial route. Ten rabbits inoculated in this manner showed no local or systemic adverse reactivities, exhibited normal weight gain and no lesions were found in the brain. In mice, in which histopathology was performed, there was no indication of encephalopathy caused by ALVAC-RG (vCP65) in the sections observed. Only very high doses of ALVAC were found to be lethal by i.c. inoculation in young adult or newborn mice further suggesting the lack of neurovirulence of the ALVAC virus[18].

After inoculation by the i.d. route, reactions were evident at the site of inoculation in mice, guinea pigs, and rabbits. In mice, the reactions consisted of small necroses, a few millimeters in diameter, at the site of inoculation. These were evident by 1 day post-
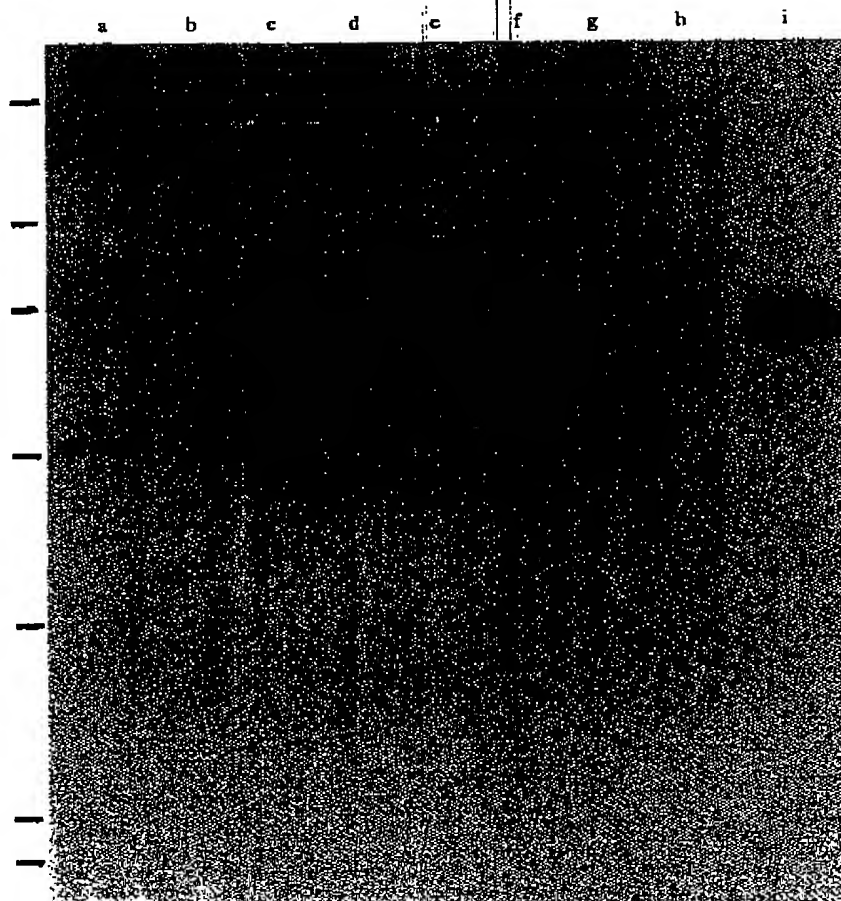
Figure 1 Immunoprecipitation analysis of expression of the rabies glycoprotein in avian and non-avian cells inoculated with ALVAC-RG (vCP65). Dishes of each cell line were inoculated at an input multiplicity of 10 p.f.u./cell with ALVAC or ALVAC-RG in the presence of ^{35}-S methionine as described in Ref. 18. Lanes a, d, g, uninfected cells; lanes b, e and h, ALVAC infected cells; lanes c, f and i, ALVAC-RG (vCP65) infected cells. Lanes a, b and c, CEF cells; lanes d, e and f, HNK cells; lanes g, h and i, HEL cells. Molecular weight markers are shown to the left of lane a and indicate migration distances for standard proteins with molecular weights (from the top) of 200, 97.4, 68, 43, 29, 18.4 and 14.3 kDa

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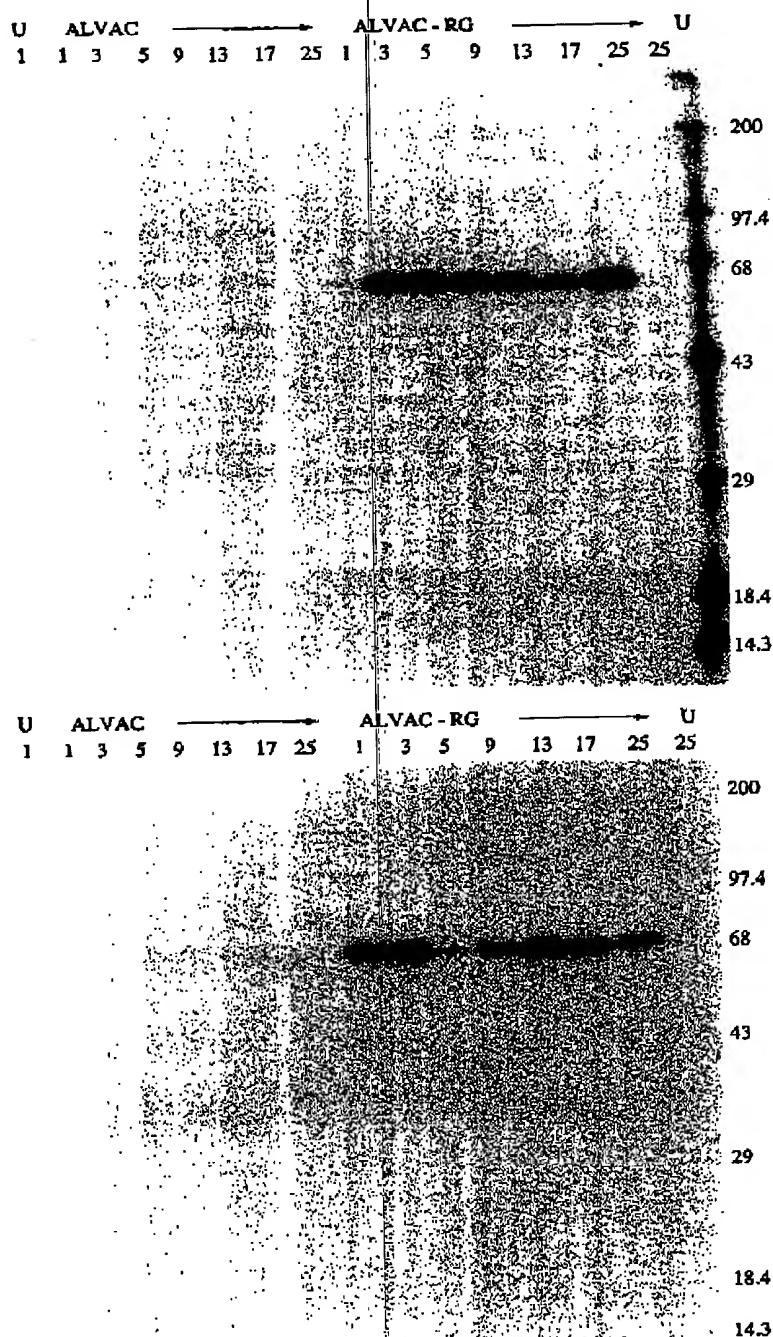
Figure 2   Time course of analysis of expression of the rabies G protein in avian and human-derived cells inoculated with ALVAC-RG. Dishes of CEF or MRC-5 cells were inoculated at an input multiplicity of 10 p.f.u. per cell with ALVAC or ALVAC-RG and labelled for 1 h with ^{35}S-methionine at appropriate times as described in Materials and Methods. Immunoprecipitation was performed as described previously[15]. Figure 2a illustrates analysis of CEF cells and 2b illustrates analysis of MRC-5 cells. U denotes an uninfected control cell lysate. ALVAC or ALVAC-RG indicates cells were infected with ALVAC parental virus or ALVAC-RG, respectively. Figures at the top of each lane indicate time of the labelling period in hours post-infection. Molecular weight markers are shown at the right of each figure (a and b) for migration of standard proteins as described in the legend to Figure 1

inoculation persisting approximately 7 days. In guinea pigs, there was a more inflammatory reaction which consisted of erythema, then a small pustule followed by necrosis. In rabbits, an inflammatory reaction was also seen which progressed to a small pustule with necrotic patches. In both guinea pigs and rabbits the dermal lesions were resolved by 21 days post-inoculation. In a similar experiment, serial dilutions of ALVAC-RG (vCP65) and KANAPOX, the original vaccinal strain of canarypox from which ALVAC was derived, were inoculated by the i.d. route into rabbits and the reactogenicity assessed. Both ALVAC-RG (vCP65) and Kanapox induced some erythema and edema with slight necrosis at the inoculation site indicating the reactogenicity was similar with the parental strain and ALVAC-RG recombinant virus. The reactivity was dose related being most pronounced with the undiluted preparation which contained $6.2 \log_{10}$ $TCID_{50}$ per ml. It should be noted that viral preparations used in these experiments were not gradient purified and the presence

of cellular components may have contributed to the reactogenicity seen following injection by the i.d. route.

Inoculation of canary birds with ALVAC-RG. In order to confirm that the virulence of the parental strain had not been altered by deletion of the C5 ORF and insertion of a heterologous coding sequence (the rabies G gene), reactogenicity of Kanapox and ALVAC-RG (vCP65) was compared in canary birds. No deaths occurred in any of the birds and body weights varied within physiological limits throughout the experimental period. One bird in each group inoculated with $5.0 \log_{10}$ p.f.u. of ALVAC-RG or Kanapox showed mild inflammation at the application site during the second week post-inoculation with redness and some swelling. All birds inoculated with $7.0 \log_{10}$ p.f.u. of either virus developed a typical pox-like take on day 5 with inflammation, swelling, a small pock, and in one bird inoculated with ALVAC-RG (vCP65), a patch of
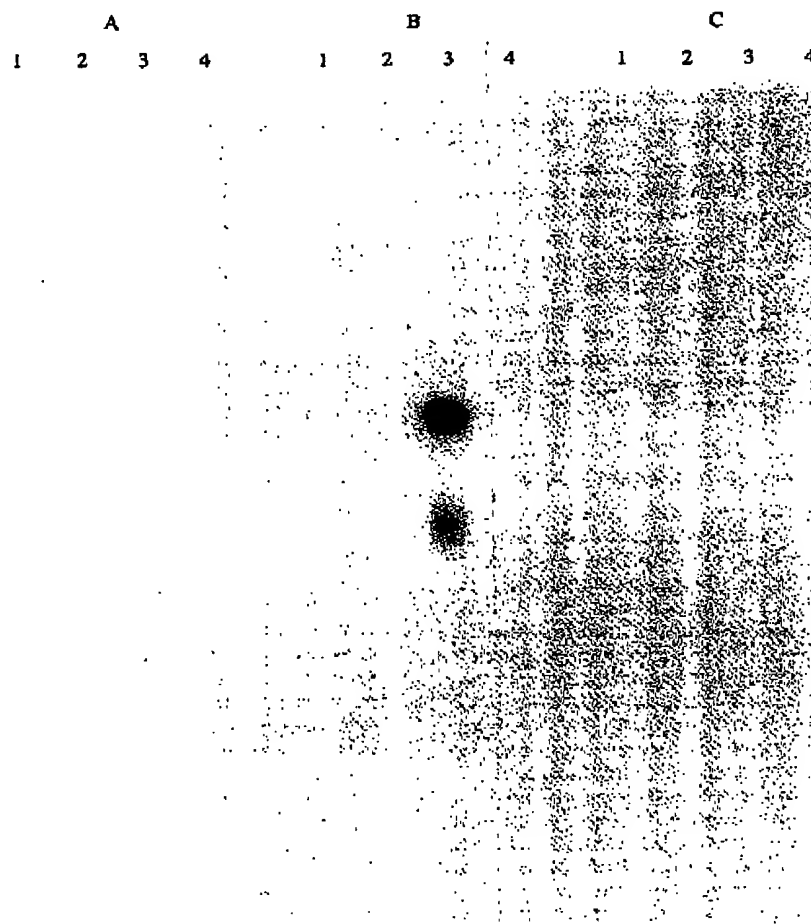
Figure 3   Analyses of viral-specific DNA accumulation in avian and human derived cell lines inoculated with ALVAC. Dishes of each cell line were inoculated and processed as described in Materials and Methods. Panel A, DETROIT 532 cells; Panel B, CEF cells, Panel C, WISH cells. In each panel, lane 1 represents uninoculated cells, lane 2 represents ALVAC-infected cells harvested at zero time, lane 3 represents ALVAC-infected cells harvested at 72 h and lane 4 represents ALVAC-infected cells incubated in the presence of 40 μg ml⁻¹ of AraC and harvested at 72 h

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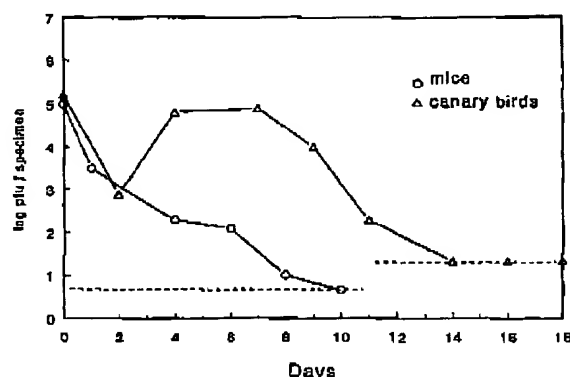
Figure 4 Analysis of virus isolation from skin of canaries and mice inoculated with ALVAC-RG. Mice and canaries were inoculated with ALVAC-RG by the i.d. route as described in Materials and Methods. At time intervals after inoculation animals and birds were sacrificed and the skin surrounding the site of inoculation was dissected, homogenized and assayed for the presence of infectious virus by titration on QT35 cells

necrosis. All lesions remained localized and were resolved by 21 days post-inoculation.

A further experiment was performed to follow the fate of inoculated virus in canary birds and in mice. Mice were inoculated by the i.d. route in the ear pinna and canary birds by smearing virus onto an area of skin on the back of the bird from which feathers had been plucked. The results of virus isolation from the area of inoculation are shown in *Figure 4*. The results indicate that in canary birds, there was an initial eclipse phase of 2 days after which an increase in infectious virus was observed for up to 7 days. Viral recovery then declined and after 14 days no virus was detected. In contrast, from day 0 the amount of virus recovered from mice progressively declined until reaching the limit of detection (20 p.f.u. per sample) on day 10 after inoculation.

In vivo studies: inoculation of non-human primates

Safety and immunogenicity in squirrel monkeys. Three groups of four squirrel monkeys (*Saimiri sciureus*) were inoculated as described in MATERIALS AND METHODS and *Table 2*, and monitored for reactogenicity as well as immune response. Animals received either ALVAC parental virus (animal Nos 22, 51, 36, and 52), ALVAC-RG (vCP65 animal) (Nos 37, 53, 38, and 54) or a canarypox recombinant expressing an Env gene product derived from an endogenous FeLV provirus (animal Nos 39, 55, 40, and 56). The initial inoculation was performed by ocular, oral and i.d. routes. No reactions were seen following inoculation of the three viruses except for minor skin lesions following i.d. inoculation of approximately 7.0 $\log_{10}$ p.f.u. Both body weight and temperature of all animals remained within normal limits. Virus isolation from ocular fluid, saliva and the site of i.d. inoculation was attempted for 11 days post-inoculation. Virus recovery was achievable from the inoculation site for 2 (6/6 animals) to 4 days (2/6 animals) following the i.d. administration of 7.0 $\log_{10}$ p.f.u. but not 5.0 $\log_{10}$ p.f.u. of all viruses. Virus was not recoverable from eye secretions or saliva at any timepoint (results not shown).

Analysis of post-inoculation sera by ELISA indicated that all animals inoculated with either ALVAC or an ALVAC-based recombinant developed a serological response to ALVAC (results not shown). All four animals inoculated with ALVAC-RG (vCP65) (animal Nos 37, 53, 38, and 54) developed rabies virus neutralizing antibody (*Table 3*), the level of which at 28 days was well above that considered to be a satisfactory response to rabies vaccination. It should be noted that 0.5 International Units, or a titer of approximately 1:16 is considered by the WHO to be the acceptable minimal response to rabies vaccination[27]. Six months after the primary inoculation, four monkeys which received ALVAC-RG (vCP65) (37, 53, 38, and 54), two monkeys which received ALVAC (22, 51), two monkeys which received an ALVAC recombinant expressing the FeLV env gene (39, 55), and one naive monkey (57) were inoculated with 6.5 $\log_{10}$ p.f.u. of ALVAC-RG (vCP65) by the s.c. route to monitor the immune response in the face of pre-existing ALVAC immunity. There were no adverse reactions to re-inoculation in any of the animals. At 28 weeks all previously inoculated animals showed some low level of canarypox ELISA antibody which was boosted 3–7 days after reinoculation (results not shown). Assessment of levels of anti-rabies antibody in sera of these animals is shown in *Table 3*. The four animals with prior exposure to ALVAC (22 and 51) or ALVAC-FeLV (39 and 55) and the naive animal (57) mounted a primary response with rabies virus neutralizing antibody present 7–11 days post-inoculation. Significantly, the four monkeys with prior exposure to ALVAC-RG (vCP65) showed an anamnestic response by 7 days post-inoculation.

Safety and immunogenicity in Rhesus macaques

Two macaques were initially inoculated with ALVAC-RG as described in *Table 2* by the s.c. route. No local or systemic adverse reactions to inoculation were noted. After 100 days, these animals were reinoculated by the s.c. route and an additional six animals were inoculated with a range of doses by the i.m. or s.c. routes. Sera of animals were monitored for the presence of anti-rabies neutralizing antibody in the RFFIT-test[24] and results are shown in *Table 4*. Animals 177 and 186 receiving ALVAC-RG (vCP65) by the s.c. route developed rabies virus neutralizing antibody detectable at 11 days post primary inoculation. Levels of antibody above the minimal acceptable level[27] were still present at 3 months when animals were re-inoculated and both animals responded with an increase in titer. Equivalent responses were obtained by either the s.c. or i.m. routes with a dose of either 7.0 or 6.0 $\log_{10}$ p.f.u. At a dose of 5.0 $\log_{10}$ p.f.u. only one animal (180) responded by the s.c. route.

Safety and immunogenicity studies in chimpanzees

Two chimpanzees were inoculated by the i.m. (animal 431) or s.c. (animal 457) routes with 7.0 $\log_{10}$ p.f.u. of ALVAC-RG (vCP65). At 12 weeks, both animals were re-inoculated in an identical manner. No local or systemic adverse reactions to inoculation were noted in either animal. Serological results are shown in *Table 5*. Both chimpanzees responded with the induction of

In vitro and in vivo studies of a canarypox–rabies recombinant: J. Taylor et al.

Table 3 Response of squirrel monkeys with prior exposure to ALVAC or ALVAC-based recombinants to inoculation with ALVAC-RG

Animal number	First inoc.	RFFIT titer at day post-inoculation[a]					
		0	7	28	180[d]	187	201
22	ALVAC	—	—.	—	< 16	< 16	200
51	ALVAC	—	—	—	< 16	50	158
39	ALVAC-FL	—	—	—	< 16	50	158
55	ALVAC-FL	—	—	—	< 16	50	126
37[b]	ALVAC-RG	< 16	< 16	1000	< 16	1580	3160
53[b]	ALVAC-RG	< 16	16	158	< 16	3980	3980
38[c]	ALVAC-RG	< 16	316	1000	< 50	1580	3980
54[c]	ALVAC-RG	< 16	250	1580	< 50	3980	10000
57	None	—	—	—	< 16	50	500

[a]Sera tested in an RFFI Test (Ref. 24). Titer expressed as reciprocal of the highest dilution showing complete inhibition of fluorescence.
[b]Animals 37 and 53 were inoculated with 5.0 $\log_{10}$ p.f.u. as described in Table 2
[c]Animals 38 and 54 were inoculated with 7.0 $\log_{10}$ p.f.u. as described in Table 2
[d]At day 180, all animals were inoculated with 6.5 $\log_{10}$ p.f.u. of ALVAC-RG by the subcutaneous route

rabies virus neutralizing antibody at 2–4 weeks post-inoculation and antibody titers were significantly boosted after the second inoculation at 12 weeks.

DISCUSSION

The studies described in this communication were conceived to evaluate, in some detail, the biological and immunological properties of ALVAC and derivative recombinants in non-avian species. The results provide a safety profile for the ALVAC vaccine vector and illustrate the utility of ALVAC as a general immunization vehicle in non-avian species.

Failure to demonstrate replication of ALVAC or ALVAC-based recombinants has been demonstrated on tissue culture cells of murine and feline origin (unpublished data). Further, no evidence for viral replication has been obtained following inoculation of ALVAC on a variety of human or monkey-derived tissue culture systems[18] and the inability to adapt the virus to growth on human or monkey-derived cell lines has been confirmed by serial blind passage of both ALVAC and ALVAC-RG (vCP65)[25].

Table 4 Serological response following inoculation of Rhesus macaques with ALVAC-RG

Animal No. /dose	Route 1[a]/2[a]	RFFIT titer at days post-inoculation								
		0	6	11	35	99[a]	101	105	114	128
177/7.7	s.c./s.c.[b]	—	—	16[d]	32	64	32	512	512	256
186/7.7	s.c./s.c.	—	—	128	512	256	256	512	512	256
178/7.0	s.c.					—	—	—	64	64
182/7.0	i.m.[c]					—	—	—	32	64
179/6.0	s.c.					—	—	—	64	32
183/6.0	i.m.					—	—	—	128	128
180/5.0	s.c.					—	—	—	32	32
184/5.0	i.m.					—	—	—	—	—

[a]Day of re-inoculation
[b]Subcutaneous route
[c]Intramuscular route
[d]Titers expressed as reciprocal of last dilution showing inhibition of fluorescence in RFFI test[24]

Table 5 Serological response of chimpanzees to inoculation with ALVAC-RG

Animal No. /route	RFFIT titer at weeks post-inoculation									
	0	1	2	4	8	12[a]	13	15	20	26
431/i.m.	< 8[b]	< 8	8	16	16	16	128	256	64	32
457/s.c.	< 8	< 8	32	32	32	8	128	512	128	128

Animals were inoculated with 7.0 $\log_{10}$ p.f.u. of ALVAC-RG by the indicated route and re-inoculated in the same manner 12 weeks later
[a]Time of reinoculation
[b]Titer expressed as reciprocal of last dilution showing inhibition of fluorescence in an RFFI test[24]

In vitro and in vivo studies of a canarypox—rabies recombinant: J. Taylor et al.

On human-derived cell cultures, no accumulation of ALVAC-specific viral DNA was demonstrated suggesting that the block to viral replication in these cell substrates occurs early in the replication cycle prior to viral DNA replication. Similar analyses performed with ALVAC-infected VERO cells have demonstrated low, but detectable, levels of accumulated ALVAC-specific DNA (data not shown). Somogyi *et al.*[28] have recently shown that in MRC-5 cells infected with fowlpox virus, both viral DNA replication and some late viral protein synthesis can be detected, albeit at reduced levels. The block in avipox productive replication in mammalian cells may vary, not only for different cell types, but also for the different avipox viruses. While the details of the molecular events responsible for the block to viral replication in non-avian species remain to be elucidated, it is significant that the expression of at least some avipox virus genes, and of appropriately regulated extrinsic immunogens, occurs in all non-avian tissue cultures tested. Additionally, when the time course of expression of the rabies G gene was monitored in these cells, it was evident that expression could be detected continuously from 1 to 25 h post-infection when the experiment was terminated (*Figure 2*).

Previous *in vivo* studies in a variety of species including mice, cats, and dogs[2,3] had shown no reactogenicity following inoculation of a CPV recombinant. A number of laboratory animals were inoculated with ALVAC or ALVAC-RG (vCP65) to extend these results. Safety studies performed in laboratory animals via the s.c. and i.m. routes indicated no reactogenicity. Similarly, inoculation of mice and rabbits by the i.c. route showed no evidence of neurovirulence. This is also supported by data of LD_{50} values by i.c. inoculation of young or newborn mice[18]. Further, no adverse reactions have been observed upon inoculation of immunodeficient mice[18].

Inoculation of rabbits with high doses of CPV by the i.d. route resulted in the formation of poxvirus-like lesions. In related experiments not reported here, lesions were induced on rabbits by i.d. inoculation of 8.0 log_{10} p.f.u. of ALVAC. When the dose was reduced to 7.0 and 6.0 log_{10} p.f.u., minimal reactogenicity was apparent. Similarly, skin lesions were evident at the site of i.d. inoculation of ALVAC and derived recombinants in squirrel monkeys but only sporadic virus recovery was possible through 4 days post inoculation. The formation of a lesion at the site of i.d. inoculation may be a cytotoxic phenomenon due to expression of early viral functions or may be linked to the presence of cellular components in the inoculum. Reactogenicity by the i.d. route was not related to altered pathogenicity following insertion of a foreign gene since an equivalent effect was seen with the parental canarypox vaccine strain, Kanapox.

Three non-human primate species were inoculated with ALVAC-RG (vCP65) to monitor safety and immunogenicity. No adverse signs of infection or disease were seen in the squirrel monkeys, macaques, or chimpanzees following inoculation by a variety of routes. All three species responded with significant levels of rabies virus neutralizing antibody which were boosted after a second inoculation. Significantly, squirrel monkeys with a history of prior exposure to CPV or CPV recombinants did not show a diminution of response when inoculated with ALVAC-RG (vCP65). These monkeys have since been inoculated with a third ALVAC recombinant expressing the measles virus fusion and hemagglutinin glycoproteins and have responded with protective levels of measles virus HI antibody comparable to that induced in naive animals (unpublished data). These results indicate that prior exposure to ALVAC recombinants should not preclude subsequent vaccinations with a novel ALVAC recombinant. In addition, it should be noted that four of the monkeys (22, 37, 38 and 39) had also received vaccinia virus three months before inoculation with ALVAC or ALVAC-based recombinants. The fact that the rabies-specific immune response was not diminished in these animals may indicate that in humans, prior immunity to vaccinia virus may not limit use of an ALVAC-based recombinant vaccine. The concept of using a non-replicating vector system in humans has been demonstrated in Phase 1 clinical trials with the ALVAC-RG (vCP65) recombinant virus. Volunteers inoculated with ALVAC-RG (vCP65) demonstrated significant immune responses to the extrinsic immunogen in the absence of unacceptable local or systemic reactions to vaccination[10,11].

Practical issues of utilizing ALVAC-based recombinants for specific veterinary applications have been addressed in the target species. In a duration of immunity study, dogs inoculated with a single dose (6.7 log_{10} $TCID_{50}$) of ALVAC-RG (vCP65) were protected against a lethal challenge with rabies virus at 36 months post-inoculation (manuscript in preparation). Other unpublished studies have provided evidence that these vector systems may be useful in the presence of maternally derived antibodies (manuscript in preparation). The safety and immunogenicity profile of ALVAC-based recombinants suggests a strong potential for ALVAC as a generic immunization vehicle in other veterinary as well as human applications.

ACKNOWLEDGEMENTS

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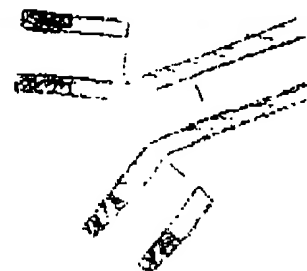For: POLYEPITOPE VACCINES

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*b*acterial Vaccine Vectors

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■

Abstract

Bacterial vaccine vectors can, in theory, be developed from any bacterial pathogen by rendering them avirulent using methods of classical or rational attenuation. *Mycobacterium bovis* BCG is an example of a vector system that was derived using the former approach whereas both *aroA Salmonella* and Toxminus *Corynebacterium pseudotuberculosis* were generated using site-specific mutagenesis. Introduction of defined mutations has the advantage over random mutagenesis because the possibility of reversion to virulence can be excluded. Vector technology has the potential to provide an oral, single dose, multivalent vaccination regime. For this benefit to be realised, in addition to achieving high levels of regulated foreign gene expression from stable loci, the commercial issues of product reactogenicity, transmission potential and ease of manufacture and storage must be addressed. With reports of protection from single dose administration of live recombinant vaccines increasing, the prospects for successful development of this technology should be regarded as high.

Introduction

Vaccine vectors are attenuated pathogens expressing foreign antigens that when vaccinated into an animal host can elicit protective immune responses. Vectors are generally derived from either pathogenic bacteria or viruses and protective antigens are cloned from a variety of pathogens including bacteria, viruses and parasites. In principle, viral and bacterial vectors are favoured for delivery of viral and bacterial antigens, respectively, but no particular preference is made where parasite antigens are concerned. Regardless of the preferred combination, the efficacy of each vector vaccine will require a complex of host immune responses which will in part, depend upon the nature of the vector, route of administration and the quantity, quality and timing of the antigen presentation. Although such general principles of immunology and molecular biology can be used as a guide for making vector vaccines, optimisation probably will require empirical approaches.

Several advantages have been proposed for live vector compared with killed vaccines (Table 1). The success of vector vaccines may well depend upon the beneficial impact these attributes have upon the final product. I review the progress made towards fulfilling these and other criteria important to the development of a commercial bacterial vector vaccine. The analysis will focus primarily upon the well-known *Mycobacterium bovis* BCG and *aroA Salmonella* vectors in addition to the more recently developed Toxminus *Corynebacterium pseudotuberculosis* system.

VACCINES IN AGRICULTURE

A. L. M. Hodgson

<table>
<tr><td colspan="2">Table 1. Advantages of bacterial vaccine vectors</td></tr>
<tr><td>Unlimited cloning capacity</td></tr>
<tr><td>Single dose vaccination</td></tr>
<tr><td>No adjuvants</td></tr>
<tr><td>Response modifiers can be delivered simultaneously</td></tr>
<tr><td>Multivalency</td></tr>
<tr><td>Ease of administration</td></tr>
<tr><td>Ultimate safety; antibiotic treatment possible</td></tr>
<tr><td>Low cost of production</td></tr>
</table>

Table 2. Host -protective toxoid vaccines

Bacterium	Toxin	Disease/Host	Reference
Corynebacterium pseudotuberculosis	Phospho-lipase D	Cheesy gland/ Sheep	Burrell (1983)
Pasteurella haemolytica	Leukotoxin	Shipping fever/ Cattle	Potter et al. (1992)
Actinobacillus pleuropneumoniae	Cytolysin	Pleuro-pneumonia/Pigs	Devenish et al. (1990)
Pasteurella multocida Type D	Dermonecrotic	Atrophic rhinitis/Pigs	Nielson et al. (1991)
Bordetella bronchiseptica	Dermonecrotic	Atrophic rhinitis/Pigs	Nagano et al. (1988)

Classical versus Rational Attenuation

Attenuation of bacterial pathogens has often be accomplished using conventional methods such as passage in non-host animals or through progressive *in vitro* culture (Buck 1930; Lawson *et al.* 1966; Kadel *et al.* 1985; Xie 1986; Chengappa *et al.* 1988). Perhaps the classic example of the latter is the production of the attenuated form of *Mycobacterium bovis*: *Mycobacterium bovis* BCG. From 1908 to 1921 the French scientists Calmette and Guerin made 231, three-week subcultures on 5% glycerinated beef-bile potato media of a virulent isolate of *Mycobacterium bovis*. The organism became progressively attenuated as demonstrated in successive experiments on different animals and no reversion to virulence was detected during the course of intensive investigations conducted between the mid 1920s and 1930 (reviewed by Collins and Grange 1983; and Lugosi 1992). The effectiveness of *in vitro* passage as a means of attenuation was further demonstrated by the emergence of BCG substrains displaying reduced levels of the original residual virulence that developed as a consequence of subculturing BCG in different laboratories (Sher *et al.* 1973; Lugosi 1992). BCG has now been given to nearly 3000 million people and has a case fatality rate of about two per 10 million (Cryz 1992).

Contrasted to classical attenuation is the more recent concept of rational attenuation. The difference between the approaches is that in the classical process a reduction in virulence is measured, then the genetic basis for the attenuation determined (if possible) and in rational attenuation a mutation is targeted to a specific gene and the outcome assessed. The mutations responsible for attenuating *Mycobacterium bovis* BCG are not known; however, complementation studies using cosmid libraries in a bid to restore virulence are currently being conducted to help identify the inactivated genes. Live vaccines or vectors produced by introducing non-revertible, defined attenuating mutations must be regarded as safer than those derived by random mutagenesis until a substantial safety record (as with BCG) can be established.

Two of the most popular forms of rational attenuation involve making auxotrophic mutants which produce pathogens crippled in their capacity to replicate *in vivo* or which strike directly at one or more major virulence factors. Rational attenuation of *Salmonella* species has generally been accomplished using the former method, thus producing a series of possible bacterial vectors (reviewed by Curtiss 1990; Cárdenas and Clements

1992; Hodgson and Radford 1993) the archetype of which is *aroA Salmonella typhimurium* (Hoiseth and Stocker 1981). The *aroA* mutation blocks the capacity of the *Salmonella* to synthesise 2,3 dihydroxybenzoic acid thus enterochelin and *para*-aminobenzoic acid (PABA), therefore folate (Cárdenas and Clements 1992). Since neither of these precursor compounds are present in mammalian tissues, bacterial replication *in vivo* is restricted upon exhaustion of endogenous supplies (Clements 1987; Pittard 1987). The inability to synthesise PABA is probably the most important attenuating characteristic, as a mutation solely destroying this capacity is equally effective as *aroA* in attenuating *Salmonella* (Stocker 1990). Stocker (1990) speculated that ultimately, the blocking of PABA (thus folic acid synthesis) restricts persistence *in vivo* by preventing production of fMet-tRNA$^{met}_{f}$ a molecule required by the bacteria to initiate new protein chains and again, not available within the host animal.

The confidence in the effect of the *aroA* mutation on bacterial pathogenesis is perhaps reflected by the extent to which this mutation has been applied to other species of bacterial pathogens, for example *Aeromonas salmonicida* (Vaughan *et al.* 1990), *Yersinia enterocolotica* (O'Gaora *et al.* 1990), *Bordetella pertussis* (Roberts *et al.* 1990) and *Shigella* (Verma and Lindberg 1991). As predicted, all were attenuated. The *aroA* genes of *Mycobacterium tuberculosis* have been cloned (Garbe *et al.* 1990) consequently raising the possibility of deriving a defined attenuated mutant. However, this will be contingent upon developing a means of achieving site-specific mutagenesis within *Mycobacterium tuberculosis*.

Another rational method of attenuating bacterial pathogens is to delete major virulence factors. Toxin genes are particularly good candidates. Strong evidence to suggest that a toxin-negative derivative of a particular pathogen will be attenuated is provided when it can be demonstrated that toxin neutralising antibody responses are protective. Several toxoid vaccines have been shown to protect animals from homologous bacterial challenge (Table 2), thus these pathogens are good possibilities for vaccine vector development. We have proved the principle for one of these pathogens by demonstrating that site-specific deletion of the *Corynebacterium pseudotuberculosis* phospholipase D (PLD) gene (Hodgson *et al.* 1990) removes the capacity of the bacterium to produce caseous lymphadenitis (cheesy gland) in sheep (Hodgson

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Table 3. Important attributes of bacterial vector vaccines

Safety
Genetic stability
Protection from a single dose
Effective vaccination achievable using mucosal or
   parenteral administration
Low reactogenicity
No spread to secondary hosts
No adverse effects if the host has had prior exposure to
   the vector
Absence of antibiotic resistance genes
Multivalency
Easy to produce and store

et al. 1992). One of the additional benefits of producing vaccine vectors by deleting a toxin gene is the opportunity to reintroduce into the bacterium a genetically toxoided analogue. Since the toxins/toxoids are, by definition, extremely valuable protective antigens, this approach has the potential to generate exceptionally effective live vaccines which, importantly, will lack the capability of undergoing a recombination event that may restore virulence. We are currently investigating the utility of the attenuated *Corynebacterium pseudotuberculosis* strain (Toxminus) as a vaccine vector and our progress will be summarised in the next section of this review.

Development of Bacterial Vaccine Vectors

One way to assess the progress of the vaccine vector technology is to examine the level of achievement of various criteria recognised as essential for successful vaccine development. Perceived advantages of bacterial vector vaccines have been summarised (Table 1) and the attributes considered important for developing a commercial vaccine are listed in Table 3. This list has been compiled using information from the scientific literature, industry, the commercial sector and various regulatory authorites but is not necessarily exhaustive. To reflect the current status of bacterial vector technology we have examined the most advanced vectors, *Mycobacterium bovis* BCG and *aroA Salmonella*, with respect to these criteria. *Corynebacterium pseudotuberculosis* Toxminus has been included because it is a novel vector produced in our laboratories and represents a system that is in a relatively early phase of evaluation. The following provides the detail behind the summary presented in Table 4.

Safety

The safety criterion has been subdivided into an assessment of whether the vector has the capacity to cause clinical disease in the general population or only within immuno-compromised hosts (Table 4). This aspect is likely to reflect the degree of attenuation, potential for reversion or both. While BCG has a substantial safety record (see earlier) it nevertheless can produce serious complications including disseminated infection (Lotte *et al.* 1984). This vector is,

Table 4. Achievement of important attributes of vector vaccines

Attribute	BCG	aroA Salmonella	Toxminus C. pseudotuberculosis
Safety			
Clinical disease	N	N	N
Genetic reversion	N	N	N
Immunocompromised hosts	N	Y	NT
Genetic stability			
Plasmid stability	Y	Y	Y
Chromosomal integration	Y	Y	Y
AB^R genes necessary	N	N	N
Gene expression			
High level	Y (10–20%)	Y (10–20%)	Y (1–2%)
Regulation	Y	Y	Y
Single dose vaccination			
Immunological responses	Y (Table 5)	Y (Table 5)	Y (Table5)
Protection	Y	Y	Y
Reactinogenicity			
Injected	Y	Y	Y
Oral	N	N	
Shedding			
Injected	U	U	U
Oral	P	Y	Y
Adverse effect of prior exposure			
	F	F	F
Multivalent vaccination			
	F	F	F
Production and storage			
	E	E	E

N: no; Y: yes; NT: not tested; U: unlikely; P: possible; F: further work required; E: easy to grow and produce a freeze dried product that will not require cold storage.

therefore, unlikely to be fit for use in immunocompromised hosts. In contrast, *aroA Salmonella* vectors are avirulent even in burned (Carsiotis *et al.* 1989) or irradiated mice (Izhar *et al.* 1990). *Corynebacterium pseudotuberculosis* Toxminus is incapable of producing caseous lymphadenitis in sheep (Hodgson *et al.* 1992) but its effect in immunodeficient animals has not been examined.

The reversion frequency of an attenuating mutation introduced by site-specific deletion of an entire gene (e.g. *aroA* or *pld*) can be regarded as nil. However, as an added precaution, *Salmonella* vectors, especially for human use, can be made that contain double *aro* mutations (Dougan *et al.* 1988; Jones *et al.* 1991; Hone *et al.* 1991; Tacket *et al.* 1992). A major benefit of knowing the exact nature of the attenuating mutations within *aroA Salmonella* and Toxminus *Corynebacterium pseudotuberculosis* compared with the uncharacterised, random mutations of BCG is that some estimate of possibility of a genetic reversion event can be made. Establishing a safety record (potential for reversion) through decades of vaccination trials is not a practical approach for developing new vector vaccines. One possibilty for BCG (or *Mycobacterium tuberculosis* or

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Mycobacterium bovis) would be to produce *aroA* derivatives. However, although the *aro* mutation produces extremely safe attenuated pathogens firstly, their introduction is accomplished by site-specific recombination and this is not yet possible in *Mycobacterium tuberculosis* or *Mycobacterium bovis* (Kalpana *et al.* 1991) and secondly, it is important that any mutation that robs a pathogen of its virulence does not reduce its capacity to function as a vaccine vector. Part of the reason why *aro* mutants are so safe is because they are extremely attenuated. The possible consequence of this with respect to the potential for achieving single dose vector-vaccination will be discussed below.

Genetic Stability

Reversion frequencies of mutations obviously reflects genetic stability; however, here this will relate to the stability of foreign genes and their expression within host vectors. The main ways in which antigen genes are expressed is either from plasmid vectors or from chromosomal loci. Plasmid gene expression provides the potential for high protein yields by virtue of elevated gene copy number but has the disadvantage that plasmids are often unstable particularly when expressing foreign genes. Genes can be expressed stably from chromosomal loci but the advantage of gene dosage is lost. It is the capacity of a vaccine vector system to take advantage of these expression options that can be used as another measure of the development of vector technology (Table 4). At present, regulatory authorities such as the United States Food and Drug Administration (FDA) and the Department of Agriculture (USDA) are unlikely to allow registration of vector vaccines containing antibiotic resistance genes (Gay 1993; Marcus-Sekura 1993). Thus, it will be important to achieve requisite levels of gene expression without using drug selection.

The current plasmid of choice for use in BCG is pAL5000 (Rauzier *et al.* 1988) and its derivatives (Ranes *et al.* 1990; Lazraq *et al.* 1991; Hinshelwood and Stoker 1992). Although this plasmid vector appears to be unstable in fast-growing mycobacteria in the absence of selective pressure (Snapper *et al.* 1990; Lazraq *et al.* 1991) it is very difficult to cure from BCG (T. J. Doran and A. L. M. Hodgson, unpublished 1992). The effect of foreign gene expression upon the stability of the pAL5000 replicon in BCG requires further analysis and will have to be evaluated case-by-case. Plasmid vectors are often unstable in *Salmonella*, particularly in *aroA* backgrounds (Curtiss *et al.* 1989; O'Callaghan *et al.* 1990; Salas-Vidal *et al.* 1990; Tite *et al.* 1990; Yang *et al.* 1990). One approach to this problem is to include on the plasmid a gene that complements a lethal mutation within the bacterial chromosome (Galan *et al.* 1990; Porter *et al.* 1990). Provided that the transformed bacteria cannot derive the product of the mutated gene from exogenous sources, cell survival becomes conditional upon the presence of the plasmid. This is an example of a system that does not depend on drug genes. This, however, is not plasmid stability *per se*, rather it is a means of ensuring that all of the surviving cells within a

population carry the desired plasmid. The important distinction is that with conditional lethality, if the pressure to lose a plasmid is strong enough, all of the bacteria die. The impact of this situation upon the efficacy of a live, vector vaccine is obvious.

Since the conditional lethal system depends upon the complementation of isogenic (or least defined) mutants it cannot yet be used in BCG since, as mentioned before, site specific mutagenesis is not yet possible in this host. However, if recombinant pAL5000-based plasmids are found stable enough for practical purposes, the use of the mycobacteriophage L5 gene 71 as a selectable marker (Hatfull and Sarkis 1993; Donnelly-Wu *et al.* 1993) will remove the requirement for antibiotic resistance genes. This new system is most elegant and potentially a highly significant recent development in bacterial vaccine vector technology.

Foreign gene expression in Toxminus *Corynebacterium pseudotuberculosis* can be accomplished using plasmid pEP2 (Radford and Hodgson 1991). This vector appears quite stable in Toximinus since 95% of transformants retained pEP2 following 50 generations in non-selective media (A. L. M. Hodgson, J. Krywult and M. Tachedjian unpublished 1993). However, transformants can be selected only using antibiotics.

Chromosomal integration of foreign antigens has been achieved in BCG using a vector based upon the mycobacteriophage L5 (Stover *et al.* 1991) and through a process of illegitimate recombination (Kalpana *et al.* 1991) and in *Salmonella* site-specifically following electroporation of recombination cassettes (Flynn *et al.* 1990; Strugnell *et al.* 1990; Cardenas and Clements 1993). All these processes, however, concomitantly introduce antibiotic resistance genes that are required for selection. In Toxminus *Corynebacterium pseudotuberculosis* we have devised a procedure (Figure 1) that enables antigen genes to be moved to the bacterial chromosome with a double cross-over frequency of about 0.6% without antibiotic selection (A. L. M. Hodgson, J. Krywult and M. Tachedjian, unpublished 1993). This represents a major advantage for the Toxminus system. We are currently evaluating the levels of foreign gene expression.

Gene Expression

We assume that the quantity of vaccine antigen produced within the host vector will be positively correlated with the magnitude of the resulting immunological response. Therefore, a successful vector system should be capable of high levels of foreign gene expression. With this dogma in mind several strategies have been adopted to ensure that the maximum quantity of antigen reaches the host immune system. One of the ways used to achieve this has been to express genes from powerful, regulated promoters. Heat shock promoters such as *hsp*60, *hsp*70 and *groES/gro*El. have been selected for use in BCG because of their potential to be switched on (induced) under the stress conditions of the intracellular environment of the macrophage (Aldovini and Young 1991; Stover *et al.* 1991; Winter *et al.* 1991). Using this type of expression system the C-fragment from the *Clostridium*

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Wild-type *C.pseudotuberculosis*

select kan ʳ

select kanʳ
and hygʳ

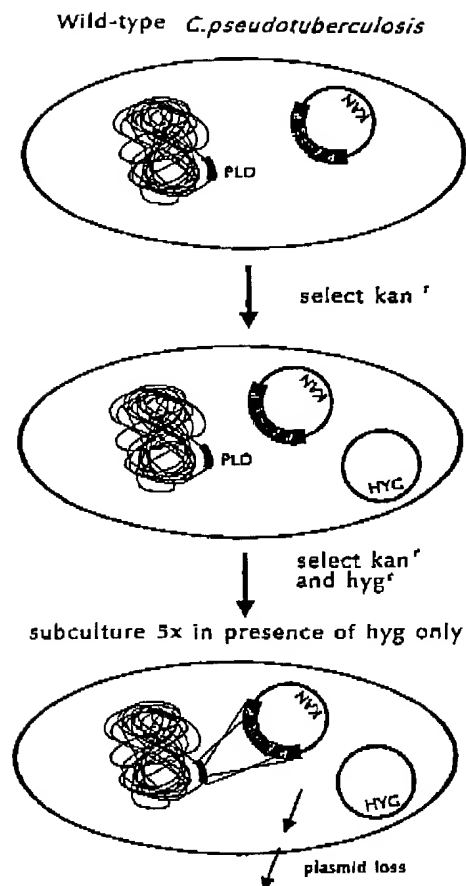subculture 5x in presence of hyg only

plasmid loss

Figure 1. Wild-type Corynebacterium pseudotuberculosis *was transformed with pEP2 carrying a recombination cassette then with plasmid pEP3 (Radford and Hodgson 1991). Transformants carrying both plasmids were resistant to kanamycin (pEP2) and hygromycin (pEP3). Loss of the pEP2 construct, thus homologous recombination with the chromosome, was promoted by repetitive subculture in media containing only hygromycin. The recombination cassette was targeted to the chromosomal PLD gene by flanking the foreign gene with PLD sequences. A double homologous cross-over event is therefore detected by choosing non-zoning colonies arising on sheep blood plates.*
PLD, *Phospholipase D gene on the bacterial chromosome;* KAN, *kanamycin resistance gene;* P, *PLD gene sequences;* E, *foreign antigen gene;* HYG, *hygromycin resistance gene.*

tetani toxin and the acquired immune deficiency syndrome (AIDS) virus HIV Nef protein have been expressed in BCG to about 0.4% (Stover *et al.* 1991) and 1%, respectively (Winter *et al.* 1991). While these levels do not seem very high, both constructs stimulated immunological responses in mice that were specific for the foreign protein. The best expression reported for BCG has been for an outer surface protein (OspA) from

Borrelia burgdorferi and the pneumococcus surface protein (PspA) that have been expressed from a pAL plasmid to between 10 and 20% of total BCG protein using the *hsp60* promoter (Langermann *et al.* 1993; Stover 1993).

Salmonella vector development has benefited from advances in *Escherichia coli* molecular genetics, consequently mechanisms of gene regulation are more numerous for this species than for either BCG or Toxminus. The use of the anaerobically regulated *nirB* promoter has enabled *in vivo* induction of the tetanus C fragment gene and also resulted in increased plasmid stability (Chatfield *et al.* 1992). Chatfield (1993) also used the *nirB* promoter to express the (P.69) antigen from *Bordetella pertussis* to huge levels (10–20%) in *Salmonella*. Other examples of regulated gene expression in *Salmonella* include the use of Lac-based promoters and the *lacI*/operator/IPTG (isopropylthiogalactosidase) inducer system. Although this form of expression may not be useful to control gene expression within the host animal, it is a very powerful research tool having been used to enable foreign proteins to accumulate to around 1.0% of total *Salmonella* protein (Schödel and Will 1990; Fairweather *et al.* 1990; Schorr *et al.* 1991). We have modified the *lac* promoter-operator system for use in Toxminus by modifying the gene encoding the *lacI* repressor protein (Pat. pending; application number PL8528/93). Lac-based promoters function constitutively in Toxminus, often producing a lethal combination with foreign genes (A. L. M. Hodgson, unpublished 1992). We hypothesised that *lacI* was not expressed well enough in Toxminus to repress gene expression so replaced the *lacI* promoter, ribosome binding site and ATG with those from the *Mycobacterium leprae* 18 kDa gene (Booth *et al.* 1988) which is known to express well in Toxminus (A. L. M. Hodgson *et al.* unpublished 1992). In the absense of the chemical inducer IPTG and presence of the new *lacI*[18k] gene, foreign gene expression directed by the *Escherichia coli* promoter P*trc* was undetectable thus indicating very tight repression (A. L. M. Hodgson *et al.* unpublished 1993). Using this gene regulation system we have expressed an antigen from the protozoan parasite *Babesia bovis* to about 1–2% of total Toxminus protein (A. L. M. Hodgson and K. Lund, unpublished 1993).

The question of how much antigen needs to be expressed within the bacterial host cell must ultimately be judged by whether protective immunological responses can be elicited by vaccination, preferably using a single dose (Tables 3 and 4). Adding to the complexity of this issue is that since each protein will have a different set of genetic and immunological characteristics, a single rule for achieving 'useful' levels of gene expression is not possible; rather, gene expression must be approached case-by-case. To begin, the native gene is expressed in the vector, preferably under the control of a regulatable promoter to establish the natural threshold of expression. This threshold will be governed by what is loosely referred to as the 'toxicity' of the foreign protein to the host cell. Heterologous gene expression can have detrimental effects on the host bacterium for several reasons. For example, rare codons within the gene may exhaust limited supplies of specific tRNAs which

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Table 5. Example of responses to bacterial vector vaccines expressing recombinant antigens
Responses are recorded in mice unless otherwise indicated; the three bacterial vectors are *aroA Salmonella typhimurium*,
Mycobacterium bovis BCG and Tox¯ *Corynebacterium pseudotuberculosis*

(Inoc. route) No. doses	HA	CA	PA,B	Reference	(Inoc. route) No. doses	HA	CA	PA,B	Reference
aroA Salmonella typhimurium					Pertussis P.69				
Rotavirus VP7					(Oral)2	+	+	+	Strugnell et al. (1992)
(I/V)2	–	NT	NT	Salas-Vidal et al. (1990)	(I/V)2	—	+	+	
Galactosidase					*Mycobacterium bovis BCG*				
(I/V)1	+	+	NA	Brown et al. (1987)	*Galactosidase*				
Escherichia coli K88					(I/V)1	+	+	NA	Stover et al. (1991)
(I/V)1	+	NT	+	Dougan et al. (1986)	(I/D)1	+	+	NA	
(Oral)1	+	NT	LDC		(I/P)1	+	+	NA	
Leishmania gp63					Tetanus toxin C fragment				
(Oral)2	LD	+	+	Yang et al. (1990)	(I/V)1	+	—	NT	
Influenza nucleoprotein					(I/D)1	+	—	NT	
(I/V)1	+	LD	NT	Tite et al. (1990)	(I/P)1	+	—	NT	
(S/C)1	+	+	NT		HIV-1 gp120				
(Oral)1	+	+	—		All routes	—	—	NT	
Dengue 4 Env.					HIV-1 Gag				
(Oral)	LD	—	—	Cohen et al. (1990)	(I/V)1&2ᴬ	+	+*	NT	Aldovini & Young (1991)
Tetanus toxin C fragment					Env				
(Oral)2	+	NT	+	Fairweather et al. (1990)	(I/D)1	—	NT	NT	
(I/V)2	+D	+	+		(I/V)1	+	NT	NT	
Hepatitis B, Polio VP1					(I/D)1	—	NT	NT	
(I/V)2	+	NT	NT	O'Callaghan et al. (1990)	HIV-1 Nef				
*Hepatitis B*G					(S/C)1	NT	+	NT	Winter et al. (1992)
(Oral)4F	+	NT	NT	Stocker (1990)	*Galactosidase*				
(I/M)5	+	NT	NT		(S/C)1	—	+	NT	Murray et al. (1992)
*Cholera toxin subunit*E					(I/V)4	+	NT	NT	
(Oral)3	—	NT	NT		*ospA* Borrelia burgdorferi				
(I/M)3C	+	NT	NT		(I/N)	+	+	+	Langermann et al. (1993)
Streptococcus pyogenes M protein E					Tox¯ Corynebacterium pseudotuberculosis				
(Oral)3	—	NT	NT		Taenia ovis 45W				
(I/M)3	+	NT	NT		(S/C)1I	—	NT	NT	Hodgson et al. (unpub.)
HIV gp160 E					Dichelobacter nodosus Protease fragment				
(I/M)3H	+	NT	NT		(S/C)1I	+	NT	NT	
(Oral)5	—	NT	NT		Anaplasma marginale (antigen)				
Escherichia coli enterotoxin					(S/C)1I	+	NT	NT	
(Oral)2	+	NT	NT	Clements et al. (1986)	Mycobacterium leprae 18kDa				
Pertussis toxin S1 subunit					(S/C)1I	+	NT	NT	
(Oral)3	+	NT	NT	Walker et al. (1992)	Corynebacterium pseudotuberculosis Phospholipase D toxoid				
					(Oral)1	+	NT	+	
					(S/C)1I	+	NT	NT	

Table 5 — continued next column

NT, not tested; NA, not applicable; S/C, subcutaneous; I/M, intramuscular; I/V, intravenous; I/P, intraperitoneal; I/N, intranasal; I/D intradermal.
A H, humoral; C, Cellular; P, protection; B any protective response is designated as positive; C limited data presented; D higher titre obtained with I/V
inoculation; E epitopes of the antigen in a flagellin gene; F rabbits, guinea pigs and mice; G rabbits and mice; H rabbits only; I sheep.
* Two doses gave a cell mediated response.

contributes to the general principle of metabolic load; the foreign protein may have a function (e.g. a protease) that harms the cell or the protein may have a damaging cellular location, for example, within the cell membrane. It is possible to increase the natural threshold of expression of some proteins by altering the codon usage of the gene to suit the host vector (Makoff et al. 1989) but, no doubt, there will be genes that cannot be expressed to useful levels within foreign hosts. One option then would

be to explore the possibility of isolating specific protective epitopes and delivering them to the host within highly expressed proteins (Stocker 1990).

Single Dose Vaccination

Having accepted that the ultimate test for a vector vaccine is its ability to elicit protective immune responses from a single dose, this requirement can then be used as

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another index to assess the progress of this technology (Table 4). Reflecting the number of laboratories working in the area, *aroA Salmonella* has had by far the greatest evaluation in animal vaccination trials. Protection following single dose vaccination using recombinant *Salmonella* has, however, not been common and the requirement in many cases for multiple vaccinations to elicit detectable immune responses is disappointing (Table 5; Hodgson and Radford 1993). The reason for this poor performance is not clear but factors involved in gene expression and stability as discussed above may play a role. Another issue that has been raised for the *aroA* vectors is the desirability of developing a live recombinant vaccine using a bacterium that is greatly restricted in its capacity for *de novo* protein synthesis *in vivo*. Arguably, bacterial pathogens crippled in this way may not persist within the host for long enough to stimulate desired immune responses and in addition, delivery of protective antigens would be further reduced by an inability to maintain synthesis of the new proteins *in vivo*. However, the most important event for the development of an immune response against a foreign antigen may be the initial amount of antigen within the vector and not that produced post vaccination (Brown *et al.* 1987; O'Callaghan *et al.* 1990; Cárdenas and Clements 1993). If this is the case, then the second part of the above argument is flawed and the requirement for developing *in vivo* inducible gene expression systems becomes questionable.

Only a few studies have evaluated the immune responses elicited by recombinant BCG and Toxminus. Evidence of protection is, therefore, limited (Table 5) but recent high level expression of the *ospA* gene from *Borrelia burgdorferi* in BCG and the preliminary report of protection following challenge is very promising (Langermann *et al.* 1993; Stover 1993). Probably the most important result that we have had so far using the Toxminus vector is the protection of sheep from caseous lymphadenitis using a single, oral dose of Toxminus expressing a genetic toxoid of the *Corynebacterium pseudotuberculosis* PLD gene (Table 5). Interestingly, in contrast to subcutaneous inoculation (Hodgson *et al.* 1992), sheep vaccinated orally with Toxminus alone were not protected (A. L. M. Hodgson, M. Tachedjian, L. A. Corner and A. J. Radford unpublished 1992). Protective immunity was, therefore, clearly a consequence of the immune response to the vectored PLD protein. This result establishes the strong potential of the Toxminus system as a vaccine vector.

Other Issues

Vaccine reactivity is an important consideration for humans and in production animals, because carcase damage reduces meat value. Site reactions following subcutaneous injection of BCG are well known and are a side effect of its powerful immunostimulatory properties. *Corynebacterium pseudotuberculosis* has a cell wall that is chemically related to BCG (Batey 1986) and as a consequence produces a similar type of inflammatory response upon injection. Toxminus appears to be a little more reactive in sheep than cattle causing swelling at the

site of subcutaneous inoculation of 10^5 bacteria that resolves within about seven days (A. L. M. Hodgson and A. J. Radford 1991). Bacterial vectors will require studies to determine the minimum doses required to stimulate protective immune reponses and then an assessment can be made as to the acceptability of the resulting site reactions. Site reactions can be avoided altogether using oral or intranasal (mucosal) vaccination and significant immunological responses have been measured for all three vectors using this route (Table 5). The choice of the route of vaccination is not only influenced by site reactivity, for example, the chance of transmitting the vaccine vector is likely to be higher for animals vaccinated orally than subcutaneously due to the prospect of faecal shedding. Parenteral vaccination using *aro*-dependent *Salmonella* has been used to reduce faecal shedding from pigs and chickens challenged orally with virulent *Salmonella* (Lumsden *et al.* 1991). Part of the concern with transmission of live recombinant vaccines lies with the movement of attenuated pathogens and their genetic material into non-target species where risk assessments have not been made, and with the possibility of eliciting undesirable or unwanted immunological responses. *aroA-aroD Salmonella* were shed from calves orally dosed with 10^{10} live organisms (Jones *et al.* 1991) and we detected Toxminus in the faeces of one sheep from 18, post administration of 10^{10} Toxminus in a saline drench (A. L. M. Hodgson, M. Tachedjian, L. A. Corner and A. J. Radford 1992). The practical possibility that shedding could result in transmission will need a detailed investigation for all prospective live vaccines.

The route of vaccination can influence the type of immune reponse elicited. Generally, oral and subcutaneous vaccinations are favoured when a secretory IgA or a cellular response are required, respectively. More detailed studies of the immunological responses generated following vaccination using live delivery vehicles will establish the best match of vector and route of administration to provide protection against specific diseases.

An issue that has been raised regarding the consequences of repetitive vaccination using live vectors is immunological tolerance. Thus far there is no evidence that multiple use of vector vaccines reduces efficacy. Immunological experience with homologous and heterologous *Salmonella* actually potentiated antibody response to subsequent vaccinations (Bao and Clements 1991) and several studies have used booster immunisations to enhance the immunological reponse to a vectored antigen (Table 5). Although it is not generally considered that repetitive vector vaccination will result in reduced efficacy (Cárdenas and Clements 1992) the requirement for multiple immunisations would be reduced by producing multivalent vaccines. Bacterial vector vaccine technology is still at the stage of proving the principle for single antigens, so there is little information on multivalency for expressing more than one protective antigen simultaneously and then assessing the protective immune response. The prospects for developing multivalent vaccines should be regarded as very good given that once protective immunity is established from an expressed foreign antigen, a bivalent vaccine is possible. For

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example, *Salmonella typhi* (Ty21) expressing cholera toxoid (Cryz 1992) could protect against cholera and typhoid. Once approaches toward multivalency become more sophisticated, issues such as antigenic competition will have to be addressed.

Conclusions

1. Bacterial pathogens can be attenuated for use as vaccine vectors using classical methods such as repetitive culturing *in vitro* or rationally, by site-specific deletion of major virulence determinants or genes within essential biochemical pathways.

2. Rational attenuation is the method of choice since it avoids the possibilty of reversion, is relatively fast to accomplish and can be applied, with a high probability of success, to several bacterial species.

3. Attenuation must find a balance between pathogenicity and innocuity so that a given vector retains sufficient capacity to persist within the host long enough to stimulate protective immune responses.

4. One of the most important scientific goals for the development of a successful bacterial vector vaccine is to achieve high level, regulated gene expression.

5. Important commercial aspects that must be addressed are product safety, stability, reactivity, transmissability and ease of manufacture and storage.

6. Even though the most advanced vaccine vectors have not yet fulfilled all of the above scientific and commercial criteria, recent data indicate that vector vaccines are progressing well and should have a strong probabilty of becoming a practical reality.

Acknowledgments

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PATENT

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DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations

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ABSTRACT   Plasmid DNAs expressing influenza virus hemagglutinin glycoproteins have been tested for their ability to raise protective immunity against lethal influenza challenges of the same subtype. In trials using two inoculations of from 50 to 300 μg of purified DNA in saline, 67–95% of test mice and 25–63% of test chickens have been protected against a lethal influenza challenge. Parenteral routes of inoculation that achieved good protection included intramuscular and intravenous injections. Successful mucosal routes of vaccination included DNA drops administered to the nares or trachea. By far the most efficient DNA immunizations were achieved by using a gene gun to deliver DNA-coated gold beads to the epidermis. In mice, 95% protection was achieved by two immunizations with beads loaded with as little as 0.4 μg of DNA. The breadth of routes supporting successful DNA immunizations, coupled with the very small amounts of DNA required for gene-gun immunizations, highlight the potential of this remarkably simple technique for the development of subunit vaccines.

Gene vaccines, or the use of antigen-encoding DNAs to vaccinate, represent a new approach to the development of subunit vaccines (1–4, 29). A subunit vaccine presents only selected components of a virus to the immune system. Prior methods of subunit vaccination have used purified proteins or viral vectors. Each of these methods has substantial limitations that would be overcome if the immunizing protein (and only the immunizing protein) could be expressed in host cells. Gene vaccines offer this opportunity, with immunization being accomplished by host cells taking up and expressing an inoculated DNA.

In this paper we evaluate how the route of DNA inoculation affects the ability to raise protective immunity. In undertaking the study, we assumed that the efficacy of different routes of DNA immunization would reflect both the efficiency of *in vivo* transfection (DNA uptake and expression) and the efficiency with which transfected cells presented proteins to the immune system. Studies in rodents on the transfection efficiency of injected DNA have demonstrated that muscle is 100–1000 times more permissive than other tissues for the uptake and expression of DNA (5–8). Tissues also differ in the efficiency with which they present antigens to the immune system. Tissues, such as the skin and the mucosal linings of the respiratory tract and the gut, that serve as barriers against the entry of pathogens have associated lymphoid tissues that provide high levels of local immune surveillance (9–15). Such tissues also contain cells that are specialized for major histocompatibility class II-restricted presentation of antigens to T-helper cells. T-helper cells produce the lymphokines that induce growth and differentiation of lymphoid cells. In view of the above, DNA

inoculations were undertaken (*i*) by a route that supports unusually efficient transfection (muscle), (*ii*) by routes that support less efficient transfection but represent routes frequently used for the administration of an antigen to a test animal (subcutaneous, intraperitoneal), and (*iii*) by routes that support less efficient transfection but deliver DNA to tissues with high levels of local immune surveillance (skin and respiratory passages).

The effect of the route of inoculation on DNA vaccination was evaluated in murine and avian influenza virus models. In both models, the vaccine consisted of purified plasmid DNA that had been designed to express an influenza virus hemagglutinin glycoprotein. This glycoprotein mediates adsorption and penetration of virus and represents a major target for neutralizing antibody (16, 17). A number of antigenically distinct subtypes of hemagglutinin glycoproteins are found in naturally occurring influenza virus infections (18). In the murine model, plasmid DNA expressing the hemagglutinin subtype 1 (H1) protein was used to protect against a lethal challenge with a mouse-adapted influenza virus with an identical H1 gene. In the chicken model, DNA expressing the hemagglutinin subtype 7 (H7) protein was used to vaccinate against a lethal H7 virus with an antigenically distinct H7 glycoprotein (19).

MATERIALS AND METHODS

Vaccine DNAs. Plasmids pCMV/H1 and pCMV/H7 were constructed by substituting cDNAs for H1 (20) or H7 (21) for interleukin 2 (IL-2) sequences in the pBC12/CMV/IL-2 expression vector (22). Substitutions were accomplished by blunt-end ligations into the ≈4.0-kb *Hind*III-*Bam*HI fragment of pBC12/CMV/IL-2. Constructs in the correct orientation to express the H1 or the H7 gene under the control of the cytomegalovirus (CMV) immediate early promoter were identified by restriction endonuclease digestions. Expression of H1 or H7 was confirmed by indirect immunofluorescent staining of transiently transfected COS cells. The pCMV/control plasmid was generated by deleting a 0.7-kb DNA fragment containing the gene for IL-2 from pBC12/CMV/IL-2. p188 DNA represents a previously constructed DNA that uses retroviral transcriptional control elements to express H7 (4). pRCAS, a replication-competent retroviral vector from which the replication-defective p188 was derived, served as a control DNA (23). DNAs were grown in *Escherichia coli* DH5 bacteria and purified on cesium chloride density gradients by standard protocols. DNA concentration was determined by optical density at 260 nm and confirmed by comparing intensities of ethidium bromide-stained restriction endonuclease fragments with standards of

known concentration. DNA was stored in 1 mM Tris, pH 7.8/0.1 mM EDTA. For injections, DNA was diluted in saline (0.9% NaCl).

Vaccine Trials. Vaccine trials in mice were accomplished by the administration of DNA to 6- to 8-week-old BALB/c mice. Two DNA inoculations were given, one at time 0 and the second 4 weeks later. Lethal challenge was administered at 10 days after the second DNA inoculation by inhalation of virus into the lungs of Metofane (Pitman–Moore, Mundelein, IL.)-anesthetized mice. The challenge consisted of 250 plaque-forming units [10–100 times the median lethal dose (LD_{50})] of mouse-adapted A/PR/8/34 (H1N1) influenza virus in 100 µl of saline supplemented with 0.1% bovine serum albumin. The challenge virus underwent localized replication in the respiratory tract causing death due to pneumonia within 1–2 weeks. Routes of DNA inoculation included the following: intravenous (tail vein), intraperitoneal; intramuscular (both quadriceps), intranasal (DNA drops administered to the nares of mice anesthetized with Metofane), intradermal (foot pad), and subcutaneous (scruff of the neck). In general, 100 µg of DNA was administered in 100 µl of saline per test site. For foot-pad inoculations, 50 µg of DNA was administered in 25 µl.

Vaccine trials in chickens were conducted in a U.S. Department of Agriculture-approved P3 facility. Three-week-old specific pathogen-free chickens (SPAFAS, Norwich, CT) received two DNA inoculations, one at time 0 and the second 4 weeks later. Lethal challenges were administered via the nares at 1 or 2 weeks after the second DNA inoculation. This challenge consisted of 10^4 egg infectious doses (100 LD_{50}) of A/Chicken/Victoria/1/85 (H7N7) influenza virus. The challenge infection spread rapidly throughout the internal organs and brain of chickens, causing death within 4–7 days. Routes of inoculation included the following: intravenous (wing vein), intramuscular (breast muscle); intratracheal (DNA drops administered to the trachea), subcutaneous (nape), intrabursal (injections just above the chicken's vent), and intraorbital (DNA drops administered to the eye). In general, 100 or 200 µg of DNA was administered in 200 µl of saline.

In both murine and avian trials, sera were collected immediately prior to each DNA inoculation, immediately prior to challenge, and at two times after challenge. Test animals were observed throughout the trials, and mice were weighed regularly beginning at the time of challenge.

Gene Gun-Delivered DNA. Plasmid DNA was affixed to gold particles by adding 10 mg of 0.95-µm gold powder (Degussa, South Plainfield, NJ) and an appropriate amount of plasmid DNA to a 1.5-ml centrifuge tube containing 50 µl of 0.1 M spermidine. Plasmid DNA and gold were coprecipitated by the addition of 50 µl of 2.5 M $CaCl_2$ during vortex mixing, after which the precipitate was allowed to settle and was washed with absolute ethanol and resuspended in 2.0 ml of ethanol. The gold/DNA suspension was transferred to a capped vial and immersed in a sonicating water bath for 2–5 sec to resolve clumps. Then 163 µl of the gold/DNA suspension was layered onto 1.8 cm × 1.8 cm Mylar sheets and allowed to settle for several minutes, after which the meniscus was broken and excess ethanol was removed by aspiration. Gold/DNA-coated mylar sheets were dried and stored under vacuum. The total amount of DNA per sheet was a function of the DNA/gold ratio and ranged from 0.2 to 0.0002 µg per sheet. Animals were anesthetized with 30 µl of Ketaset/Rompun (10:2). Abdominal target areas were shaved and treated with Nair (Carter–Wallace, New York) for 2 min to remove residual stubble and stratum corneum. Target areas were thoroughly rinsed with water prior to gene delivery. DNA-coated gold particles were delivered into abdominal skin with the Accell instrument (Agracetus, Middleton, WI), which employs an electric spark discharge as the

motive force (24). Each animal received two nonoverlapping deliveries per immunization, at a discharge voltage of 17 kV.

Serology. Anesthetized mice were bled from the eye vein into 40-µl nonheparinized microhematocrit tubes. Sera for members within a group were pooled. Hemagglutination inhibition assays were performed with chicken red blood cells and mouse serum that had been pretreated with kaolin to remove background activity (25). Hemagglutination inhibition titers are the reciprocal of the highest serum dilution giving complete inhibition of hemagglutination. The isotypes of mouse antibodies were determined by enzyme-linked immunosorbent assays (ELISAs) using standard protocols and microwell plates coated with purified A/PR/8/34 (H1N1) influenza virus. These assays used 1:1000 dilutions of isotype-specific peroxidase-conjugated antibodies that had been provided at titers with similar activities (Sigma Immuno-Chemicals).

RESULTS

Vaccine DNAs. Vaccine DNAs were constructed by creating plasmids that would express H1 or H7 glycoproteins in transfected eukaryotic cells. Two constructs, pCMV/H1 and pCMV/H7, placed cDNAs for H1 or H7 under transcriptional control elements found in the CMV immediate early promoter and the rat preproinsulin gene (Fig. 1) (22). A third construct, p188, used retroviral transcriptional control elements to express H7 (4). Control DNAs for these constructs consisted of plasmid vectors with transcriptional control elements but without inserted cDNA sequences.

Inoculations of DNA in Saline. Intramuscular, intravenous, intranasal, intradermal, subcutaneous, and intraperitoneal routes of DNA administration were tested for their ability to raise protective immunity in mice. With the exception of the intraperitoneal injections, each of these routes of inoculation raised at least some protection (Table 1). The level of protection varied, with from 67% to 95% of test groups surviving. All of the survivors developed transient signs of influenza. Excellent survival occurred in groups receiving intramuscular inoculations, intravenous inoculations, or inoculations by each of three routes (intramuscular, intravenous, and intraperitoneal). The relatively mild influenza that developed in these groups was associated with ruffling of fur and transient weight loss. Good survival, but more severe influenza, occurred in mice receiving DNA intranasally. Yet poorer survival (67–75%) and more severe signs of influenza occurred in mice receiving intradermal and subcutaneous inoculations. These groups exhibited only marginal protection by the DNA inoculations. None of the mice receiving only intraperitoneal injections survived the lethal challenge. Control groups (inoculated with pCMV/control DNA or no DNA) developed severe signs of influenza with very few mice (13%) surviving the challenge. Thus, the intramuscular, intravenous, and intranasal routes of administration each provided good protection.
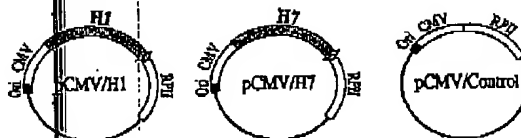

FIG. 1. Schematic of pCMV/H1, pCMV/H7, and pCMV/control DNAs used for immunizations. Ori, simian virus 40 origin of replication; CMV, CMV immediate early promoter; H1, hemagglutinin type 1 cDNA from A/PR/8/34 (H1N1) influenza virus (20); RPII, rat preproinsulin II sequences including an intron and a polyadenylylation site; H7, hemagglutinin type 7 cDNA from A/Seal/Mass/1/80 (21).

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Table 1.   Protection of mice against a lethal A/PR/8/34 (H1N1) influenza virus challenge by inoculation of pCMV/H1 DNA in saline

DNA	Route of inoculation*	Dose, μg	Signs of influenza	No. of survivors/ no. tested	% survival	Probability
pCMV/H1	i.v., i.p., i.m.	300	++	21/22	95	<0.0001
	i.m.	200	++	18/19	95	<0.0001
	i.v.	100	++	10/12	83	<0.0001
	i.n.	100	+++	13/17	76	<0.0001
	i.d.	50	++++	9/12	75	<0.001
	s.c.	100	++++	4/6	67	<0.02
	i.p.	100	+++++	0/6	0	
pCMV/control	Various	0–300	+++++	3/24	13	

See *Materials and Methods* for details on vaccination trials. Signs of influenza included weight loss, ruffled fur, and lethargy. These were scored as follows: +, transient weight loss but maintenance of smooth fur and normal levels of activity; ++, transient weight loss, some ruffling of fur and lethargy; +++, transient weight loss and more severe ruffling of fur and lethargy; ++++, more prolonged weight loss coupled with severe ruffling of fur and lethargy; +++++, weight loss and severe signs of influenza leading to death. Data are pooled from four independent trials. No data for the reported conditions have been omitted. Probability was calculated by using Fisher's exact two-tailed test comparing the frequency of survival and mortality in vaccine versus control groups.
*i.v., Intravenous; i.p., intraperitoneal; i.m., intramuscular; i.n., intranasal; i.d., intradermal; s.c., subcutaneous.

The effect of the route of DNA inoculation on vaccination was further evaluated in the chicken influenza virus model. Again, good efficacy was demonstrated for intramuscular, intravenous, and mucosal administration of the vaccine DNA (Table 2). In this highly virulent model in which vaccine and challenge H7 glycoproteins were not identical [representing genes that had undergone a 15% drift in amino acid sequence (21, 25)], groups that received DNA by multiple routes showed the best survival (50–60%). About half this level of protection (24–30%) was achieved in groups receiving DNA by only the intravenous route, only the intramuscular route, or only the intratracheal route of inoculation. Much poorer (if any) protection was achieved by subcutaneous, intraperitoneal, intrabursal, and intraorbital inoculations. Chickens receiving control DNA developed lethal influenza, with very few chickens (≈2%) surviving the challenge. Within experimental groups, surviving chickens showed more variability in the severity of influenza-related illness than surviving mice. This may have reflected the outbred genetic background of the chickens.

Gene-Gun Delivery of DNA. Gene gun-based acceleration of DNA-coated gold beads into the epidermis proved to be by far the most efficient method of DNA immunization (Table 3). The beads deliver DNA into cells, where the DNA dissolves and can be expressed (24, 26). Expression is transient (24, 26), with most of the expression being lost within 2–3 days

due to the normal sloughing of the epidermis (ref. 26 and unpublished observations). Tests of gun-delivered DNA in the murine model demonstrated that as little as 0.4 μg of DNA was sufficient to achieve 95% survival. These survivors developed very limited to no signs of postchallenge influenza. Mice receiving 0.04 μg of gun-delivered pCMV/H1 DNA had an ≈65% survival rate and suffered fairly severe signs of influenza. Mice that received 0.004 μg or 0.0004 μg of pCMV/H1 DNA succumbed to the challenge. As in tests of saline injections, mice receiving control DNA developed severe signs of influenza and had very limited survival (14%).

Antibody Responses in DNA-Vaccinated Mice. DNA vaccination by the various routes appeared to prime antibody responses. Antibody responses were assayed using tests for hemagglutination-inhibiting activity and ELISA activity (for mouse data see Table 4; for representative chicken data see ref. 4). The DNA vaccinations and booster inoculations raised only low to undetectable titers of hemagglutination-inhibiting antibodies and ELISA activity. These low levels of activity underwent rapid increases after challenge. Protection occurred in both mice and chickens that did not have detectable levels of anti-influenza antibodies before challenge. However, the best protection occurred in groups in which the DNA inoculations had raised detectable titers of antibody (Tables 1, 3, and 4).

Use of ELISAs to score the isotypes of the anti-influenza virus antibodies demonstrated that the immunizations had

Table 2.   Protection of chickens against a lethal A/Chicken/Victoria/1/85 (H7N7) influenza virus challenge by inoculation of H7-expressing DNAs in saline

DNA	Route of inoculation*	Dose, μg	No. of survivors/ no. tested	% survival	Probability
p188	i.v., i.p., s.c.[†]	300	28/56	50	<0.0001
	i.v.	100	8/33	24	<0.01
	i.p.	100	0/8	0	
	s.c.	100	0/2	0	
pRCAS	i.v., i.p., s.c.[†]	300	1/55	2	
pCMV/H7	i.v., i.p., i.m.	300	19/30	63	<0.0001
	i.m.	200	3/10	30	<0.02
	i.t.	200	3/10	30	<0.02
	i.b.	200	1/10	10	
	i.o.	200	1/10	10	
pCMV/control	Various	0–300	1/43	2	

See *Materials and Methods* for details of vaccination trials. Within experimental groups, survivors showed varying signs of influenza. Data for p188 and pRCAS DNA are pooled from five independent trials. Data for pCMV/H7 and pCMV/control DNA are pooled from seven independent trials. No data have been omitted. Probability was calculated by using Fisher's exact two-tailed test comparing the frequency of survival and mortality in vaccine versus control groups.
*i.t., Intratracheal; i.b., intrabursal; i.o., intraorbital; for others, see footnote to Table 1.
[†]Data reported in ref. 4.

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Table 3. Protection of mice against a lethal A/PR/8/34 (H1N1) influenza virus challenge by gene gun-delivered pCMV/H1 DNA

DNA	Dose, µg	Signs of influenza	No. of survivors/ no. tested	% survival	Proba-bility
pCMV/H1	0.4	±	21/22	95	<0.0001
	0.04	+++	7/11	64	<0.01
	0.004	+++++	0/5	0	
	0.0004	+++++	0/4	0	
pCMV/control	0.4	+++++	3/22	14	

See *Materials and Methods* for details of vaccination trials and legend to Table 1 for description of signs of influenza. Data are pooled from four independent trials. No trial has been omitted. Probability was calculated by using Fisher's exact two-tailed test comparing the frequency of survival and mortality in vaccine versus control groups.

primed IgG responses. Low titers of anti-influenza IgG could be detected in the sera of mice vaccinated by gun delivery or intravenous or intramuscular inoculations of DNA. Borderline to undetectable titers of IgG were present in the sera of mice receiving DNA nose drops (consistent with the poorer protection provided by this route of DNA administration). By 4 days after challenge, increased levels of IgG were detected in mice undergoing the best protection. By contrast, mice receiving control DNA did not have detectable levels of anti-influenza virus IgG until the second serum collection after challenge. This was consistent with vaccinated, but not control, groups undergoing a secondary antibody response to the challenge.

Marginal to undetectable levels of IgM and IgA were detected in both prechallenge and postchallenge sera (Table 4). The low levels of these immunoglobulin isotypes throughout the trials indicated that none of the routes of DNA inoculation were effective at raising serum IgM or IgA.

DISCUSSION

The results of our vaccine trials demonstrate that epidermal, mucosal, intramuscular, and intravenous routes of administration can be used for DNA vaccines (Tables 1–3). Our results also demonstrate that gene-gun delivery of DNA into the epidermis is a very efficient method of inoculation, achieving protection with 250–2500 times less DNA than direct inoculations of purified DNA in saline (Tables 1 and 3).

Transfection Efficiency Versus Vaccination Efficiency. One of the striking results of our DNA vaccine trials is that the efficiency of transfection does not necessarily determine the efficiency of vaccination. The high ability of rodent muscle to take up and express DNA (5–7) did not correlate with an unusual efficiency of intramuscular vaccinations (Tables 1, 3, and 4). In the mouse trials, intramuscular inoculations worked well, but no better than intravenous inoculations, and only somewhat better than the administration of DNA nose drops (Tables 1 and 4). Similar results were obtained in the chicken trials, where intravenous and intratracheal inoculations achieved levels of protection comparable with those provided by intramuscular inoculations (Table 2).

The success of DNA immunizations by the intravenous and mucosal routes may reflect efficient antigen presentation and recognition compensating for inefficient transfection. Both blood and mucosal surfaces have associated lymphoid tissues that provide specialized and highly active immune surveillance. Thus, successful vaccination by these routes may reflect a highly efficient response to very low numbers of transfected cells.

High Efficiency of Gene-Gun Vaccinations. Highly efficient immunizations were achieved by gene-gun delivery of DNA to the epidermis of mice. This method of immunization

Table 4. Antibody responses in vaccine trials testing routes of inoculation in mice

DNA and route	Time of bleed	No. tested	Titers of antibody to A/PR/8/34 (H1N1)			
			HI	ELISA value × 10⁻²		
				IgM	IgG	IgA
pCMV/H1 in saline						
i.v.	Prevac	2 (12)	<	<	<	<
	10 d PB	2 (12)	<	<	8	4
	4 d PC	1 (6)	20	<	128	4
	14–19 d PC	2 (10)	113	1	256	4
i.m.	Prevac	3 (19)	<	<	<	<
	10 d PB	3 (19)	<	<	3	<
	4 d PC	2 (13)	6	<	32	2
	14–19 d PC	3 (18)	127	<	406	2
i.n.	Prevac	3 (17)	<	<	<	<
	10 d PB	3 (17)	<	<	2	1
	4 d PC	2 (11)	<	1	2	1
	14–19 d PC	3 (17)	160	2	202	2
pCMV/control in saline various	Prevac	3 (16)	<	<	<	<
	10 d PB	3 (16)	<	<	<	<
	4 d PC	2 (9)	<	<	<	<
	14–19 d PC	1 (2)	320	<	256	<
pCMV/H1, gene gun	Prevac	2 (10)	<	<	<	<
	10 d PB	3 (16)	10*	1	10	<
	4 d PC	3 (16)	20*	2	64	<
	14–19 d PC	3 (15)	160*	<	645	<
pCMV/control, gene gun	Prevac	2 (12)	<	<	<	<
	10 d PB	3 (16)	<	1	<	<
	4 d PC	3 (16)	<	2	<	<
	14–19 d PC	1 (3)	NT	4	512	<

Data are the geometric means of the reciprocal of the final dilutions of pooled sera that scored positive for a given condition. Prevac, bleed before DNA vaccinations; 10 d PB, sera harvested 10 days after the second DNA inoculation, immediately prior to challenge; 4 d PC, sera harvested at 4 days postchallenge. No. tested, no. of groups for which pooled sera were assayed (total no. of animals contributing sera to the pools); <, activity not detected in the lowest dilution of serum used in tests [1:10 for hemagglutinin inhibition (HI); 1:100 for ELISA]; NT, not tested.
*Only one of the three pools of sera was tested for HI activity.

required 250–2500 times less DNA than the saline inoculations (0.4–0.04 µg as opposed to 100–200 µg of DNA) (Tables 1 and 3). We think the remarkable success of gene-gun vaccinations reflects the combination of efficient transfection with efficient antigen presentation and recognition. The gene gun represents a very effective method of transfecting a tissue (24, 26). When the epidermis is transfected, DNA-expressed antigens are subject to immune surveillance by the skin-associated lymphoid tissue. This lymphoid tissue is rich in cells (such as epidermal Langerhans cells) that are capable of presenting transfected antigens to the T-helper component of the immune system (12–14, 27).

Induction of Memory by DNA Vaccinations. DNA immunizations rely on low numbers of transfected, antigen-expressing cells to raise immune responses. In our trials, these low numbers of antigen-expressing cells did not induce high-titer antibody responses (Table 4 and ref. 4). However, they did prime both T-helper and B-cell memory. This memory appeared to provide protection by supporting the mounting of secondary responses in challenged animals (Table 4). Evidence for the priming of memory is provided by the DNA inoculations raising antibodies belonging to the IgG

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isotype. IgG is produced by differentiated plasma cells that have undergone immunoglobulin rearrangements in response to T-cell help (28). Evidence for the mobilization of memory in response to the challenge is found in the rapid increases in serum IgG after challenge (Table 4).

Summary. Our studies demonstrate that many routes of DNA inoculation can be used for raising protective immune responses. Two of these we consider particularly promising: (*i*) vaccination by gun delivery of DNA into the epidermis and (*ii*) vaccination by administration of DNA to mucosal surfaces. Both of these routes of administration should raise responses that will provide systemic immunity as well as specialized surveillance for major portals of pathogen entry.

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PATENT

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Exposing the Immunology of Naked DNA Vaccines

Minireview

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Vaccines represent the most commonly employed immunologic intervention in medicine today. Indeed, they are currently one of the few antigen-specific approaches with clearly documented clinical success. Current estimates by the Centers for Disease Control indicate that greater than 5,000,000 doses of vaccine against some infectious organisms are administered yearly in the United States, making vaccines the most commonly administered immunotherapeutic. Current vaccines target only a tiny fraction of infectious diseases, since prophylaxis against some of the most common and deadly infections in the third world is limited by expense and ease of distribution. In addition to the public health concerns of expense and distribution, other features of current vaccines limit their efficacy. While most current vaccines typically elicit reasonable antibody responses, cellular responses (in particular, major histocompatibility complex [MHC] class I-restricted cytotoxic T cells) are generally absent or weak. For many infectious diseases, such as tuberculosis and malaria, humoral responses have been shown to be of little protective value against infection. Another limitation of most current vaccines relates to the limited duration of immunologic memory. Ideal vaccines would provide lifelong prophylaxis, a goal generally not achieved by current formulations.

In the last three years, DNA vaccines have burst onto the scene as a radically new approach to infectious disease prophylaxis. One of the most surprising and important features of DNA immunization is that purified "naked" DNA appears to be taken up and expressed by cells in vivo with much greater efficiency than would have been predicted by the experience with DNA transfection in tissue culture. This finding provides the basis for a critical pharmaceutical advantage of DNA vaccines: namely, simplicity of preparation. In addition, naked DNA can be produced in large scale with tremendous purity, allowing for freedom from contamination with potentially dangerous agents. The final pharmaceutical advantage of DNA is its tremendous stability relative to proteins and other biologic polymers, a feature likely to be more relevant for the production of vaccines than the recreation of dinosaurs.

From an immunologic perspective, the unique ability of DNA to either integrate stably into the genome or be maintained long-term in an episomal form provides the potential for long-lived antigen expression. This feature thus has implications for the duration of immunologic memory achievable with nucleic acid vaccines. Despite the flurry of reports documenting the ability of naked DNA vaccines to induce both immunologic and protective responses in animal models, the mechanism by which DNA injections activate the immune system against the encoded antigens remains somewhat mysterious. Nonetheless, given what is now understood about pathways of antigen processing and the requirements for T cell activation, exposing the mechanisms of immune activation by naked DNA may reveal some provocative clues to how the immune system deals with different forms of antigen.

Methods of In Vivo Transduction with Injected DNA
As is so often the case with active areas of investigation, evidence for in vivo transduction by injection of purified DNA was observed many decades ago, but remained largely unnoticed until its more recent resurrection. As early as 1960, Ito (1960) demonstrated the induction of papillomas in rabbit skin by injecting phenol-extracted nucleic acids from the Shope rabbit papilloma virus. Then, in 1990, Wolff et al. demonstrated direct gene transfer into mouse muscle in vivo with reporter constructs (Wolff et al., 1990). Using either chloramphenicol acetyl transferase, luciferase, or β-galactosidase, they demonstrated that injection of either purified RNA or DNA could result in expression of the appropriate enzyme activity within the skeletal muscle. When 100 μg of purified DNA consisting of the reporter gene linked to a Rous Sarcoma Virus LTR (RSVL) promoter was injected, episomal plasmid DNA could be detected by Southern blot 30 days later and enzyme activity persisted for at least 60 days after injection. The stability of this episomal form of DNA is presumably due to the low proliferative state of myocytes in vivo. Numerous subsequent studies have evaluated different parameters of in vivo gene transfer, including injection vehicle, promoter, DNA structure, and route of injection (Davis et al., 1993a; Eisenbraun et al., 1993; Fynan et al., 1993b; Jiao et al., 1992; Manthorpe et al., 1993; Prigozy et al., 1993; Wolff et al., 1991).

Surprisingly, most investigators have found that simple saline solutions appear to be quite reasonable carriers, often resulting in transfection of between 1%–5% of myofibrils in the vicinity of the injection site in the case of intramuscular administration. DNA preparations that are typically used to transfect cells in vitro, such as calcium phosphate precipitates or liposomal preparations, do not appear to enhance the efficiency of in vivo transfer (Manthorpe et al., 1993; Wolff et al., 1990). In fact, most agents tested that might theoretically enhance in vivo transfection efficiency appear to interfere with gene transfer, at least into skeletal muscle. Nonetheless, there are a few reports that coinjections of toxic agents intended to cause muscle necrosis and repair either prior to or concurrently with injection of DNA, can increase gene transfer and expression (Davis et al., 1993a, 1993b; Vitadello et al., 1994; Wang et al., 1993a, 1993b). These include local anesthetics, such as bupivicaine, and myotoxins, such as cardiotoxin. Different promoters that have been compared for efficiency of gene expression for in vivo DNA transfer include CMV-IE, RSV, SV40, SRα, actin, MCK, α-globin, adenovirus, and dihydrofolate reductase. While relatively few direct comparisons have been performed, viral promoters

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with broad cell type specificity such as the CMV and RSV promoters appear to generate the most consistently high levels of expression of reporter constructs.

With regard to site of injection, the largest experience is with injection into skeletal muscle. Other tissue types have also been shown to express gene products after DNA injection, including cardiac muscle, liver, and dermis (Acsadi et al., 1991; Malone et al., 1994; Raz et al., 1994). One of the new technologies that has engendered particular interest in intradermal DNA injections is the gene gun. The gene gun, an instrument currently not covered under the assault weapons ban, takes advantage of the ability of ballistically accelerated microscopic gold particles to penetrate cell membranes without killing the cell. By mixing these gold particles with purified DNA in the presence of a polycations such as spermadine, the nucleic acid becomes coated onto the gold particles. These DNA-coated gold particles are loaded into the gene gun and the end is abutted to a shaved area of skin. Discharge of the gene gun results in penetration of the dermis between 0.1–5 mm, depending on the chosen projectile force. This form of DNA injection has been shown to transduce cells in both the dermis and epidermis (Eisenbraun et al., 1993).

Generation of Immune Responses by Naked DNA Vaccines

It is now well established that injection of naked DNA through any of a number of routes reproducibly induces both humoral and cellular immune responses against the encoded antigens. The initial report that genetic immunization could elicit immune responses measured the induction of antibodies against human growth hormone (hGH) subsequent to ballistic injection of DNA-coated gold particles with a gene gun (Tang et al., 1992). hGH gene constructs under transcription control of either the human β-actin promoter or CMV promoter induced specific anti-hGH antibody responses. The titer of antibodies was somewhat variable and strain dependent. In addition, clear-cut booster effects of subsequent DNA immunizations were observed, akin to what is typically seen with recombinant protein immunizations.

Subsequently, Liu and colleagues demonstrated that antigen-specific CTL responses could be induced by intramuscular injection of naked DNA (Montgomery et al., 1993; Ulmer et al., 1993). They utilized an influenza A model to emphasize the advantage of a vaccine strategy that could induce CTL responses, as humoral responses to influenza A tend to be strain specific and poorly cross protective. This is because the major antibody responses are directed against the hemaglutinin (HA) antigen, which varies significantly among different influenza strains. In contrast, epitopes of the influenza nucleoprotein (NP) antigen, a major target for CTL responses, demonstrate significantly less interstrain variability. Using a plasmid

containing the NP gene driven by either an RSV or CMV promoter, they demonstrated specific CTL responses against the NP 147–155 epitope presented by the H-2K^k MHC class I molecule. Importantly, animals immunized intramuscularly with NP DNA were protected from intranasal challenge with 102.5 TCID 50 of an influenza isolate, A/HK/68, which arose 34 years after the strain from which the vaccinating NP gene was isolated (A/PR/A/34).

Importantly, CTL responses against NP were found to persist at least 13 months subsequent to the intramuscular DNA injection (Yankauckas et al., 1993). These studies, as well as analogous findings in other animal models of infectious disease, suggested that naked DNA immunization could produce long-term humoral and cellular immune responses qualitatively similar to live attenuated vaccines but without the safety hazards of inoculation of live virus. Other infectious disease models in which successful immunization and at least partial protection against viral challenge have been observed include HIV (using the gp120 or gp160 genes), bovine herpes virus (GIV gene), rabies virus (surface glycoprotein gene), and hepatitis B virus (hepatitis B surface antigen) (Cox et al., 1993; Davis et al., 1993b; Wang et al., 1993a, 1993b; Xiang et al., 1994). In addition to the nucleoprotein gene, DNA vaccinations with other influenza genes including HA and matrix protein have likewise demonstrated protective responses (Fynan et al., 1993a; Montgomery et al., 1993; Robinson et al., 1993). In essentially all cases, significant titers of neutralizing antibody are induced and, in the case of rabies virus and HIV, CTL responses were also documented.

Despite the rapidly expanding volume of reports documenting successful immunization with naked DNA vaccines, there has yet to be a direct systematic comparison of the relative potency of DNA vaccines versus other live attenuated viral or recombinant protein plus adjuvant vaccines in standardized animal models. One report did compare the relative efficacies of naked DNA injections as a function of route of inoculation. Using either the HA subtype 1 (H1) protein in a mouse model of adapted influenza virus or the HA subtype 7 (H7) gene in a chicken model of influenza, six routes of inoculation (intravenous, intraperitoneal, intranasal, intramuscular, intradermal, subcutaneous) were compared in their ability to induce both antibody responses as well as protective immunity (Fynan et al., 1993b). While intramuscular injection of DNA appeared to generate the best response, intravenous, intranasal, intradermal, and subcutaneous immunizations also induced significant protection. When ballistic inoculation of DNA-coated gold particles was evaluated, equivalent levels of protection were achieved using 2–3 logs lower total DNA dose (0.4 μg) than all of the other forms of inoculation. In contrast with what might be expected using a recombinant protein vaccine, intranasal (mucosal) DNA inoculation did not result in enhanced immunoglobulin A

Figure 1. Biopsy of Muscle after Injection with Purified DNA Encoding β-Galactosidase

Purified DNA (100 μg) encoding β-galactosidase under transcriptional control of the CMV-IE promoter was injected into the quadriceps muscle of a mouse. The muscle was biopsied 5 days later and stained with bluogal and hematoxylin and eosin. Note the expression of β-galactosidase in large proportion of myofibrils (large arrows) as well as the significant inflammatory infiltrate (small arrows).

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Immunity
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(IgA) titers but rather produced IgG responses similar to intramucosal and intravenous injections. Clearly, much additional evaluation needs to be done in developing general principles for the quantitative and qualitative aspects of DNA immunization.

Mechanisms of Initiation of Immune Responses by DNA Vaccines

As eluded to above, many of the advantages of DNA vaccines have been proposed to stem from either stable integration or extrachromosomal maintenance of the DNA within cells of the injected organ. In the case of intramuscular injections, reporter constructs appear to mark myocytes specifically as the targets of integration and gene expression (Figure 1). The previously proposed mechanisms for priming of humoral and cellular immune responses by DNA vaccines have reflected the distinct pathways of MHC class I and class II antigen processing as defined in cell culture. Thus, antibody responses have been proposed to occur when antigens encoded by the transduced myocyte are released into the circulation either via secretion or via cell death. These antigens are then taken up by macrophages and B cells, thereby initiating a T helper-dependent antibody response. Alternatively, CTL priming has been proposed to occur via endogenous proteosome-dependent processing and presentation of antigens within the transfected myocyte followed by TAP-dependent presentation on the myocyte MHC class I molecule (Figure 2A). The stability of the integrated DNA sequences would produce an essentially continuous supply of antigen to drive immune responses indefinitely, thus accounting for the long-term persistence of immune responses against antigens encoded by the injected DNA.

If antigen receptor occupancy by peptide-MHC complexes were the whole story to T cell activation and immunologic priming, these proposed mechanisms would seem the most reasonable. However, they do not account for the critical role of costimulatory signals in initiating immune responses. Indeed, an increasing body of evidence in the self-tolerance field suggests that antigen recognition in the absence of appropriate costimulatory signals results in immune tolerance (by either ignorance, anergy, or deletion) rather than activation. Thus, simple release of antigens by natural processes of cell death do not result in induction of immunity. For example, the myocyte cell death that invariably occurs after running a marathon does not produce autoimmune myocytis. By analogy, it is probably oversimplistic to propose that immune responses against antigens encoded by integrated DNA activate and propagate immune responses when transfected myocytes simply die by natural processes. Similarly, the mounting evidence for costimulatory requirement in CD8 T cell activation implies that direct presentation of endogenously synthesized antigens by MHC class I molecules of transfected myocytes might not be expected to prime CTL precursors.

A critical element to the priming of both humoral and cellular immune responses that is usually left out of the picture in discussions of naked DNA vaccines is the bone marrow-derived antigen-presenting cell (APC). Indeed, although it has never been systematically studied, sites of injected muscle clearly become infiltrated with inflammatory cells (see Figure 1). As with all other circumstances of immunologic activation, it is hard to imagine that bone marrow-derived APCs within these inflammatory infiltrates do not play an important role in the vaccine effect of naked DNA immunizations. In the case of MHC class II-restricted CD4+ T cell priming, these APCs would have the opportunity to pick up locally released antigens in the interstitial spaces and carry them to draining lymph nodes where they could be presented to both B cells and T cells.

With regard to the priming of MHC class I-restricted CD8 T cells, it may also be worth considering these infiltrating APCs as prime suspects in CTL activation (Figure 2B). Despite the earlier cross-priming experiments of Bevan in the 1970s, the notion that host-derived APCs could efficiently ingest released exogenous antigens for processing and presentation on MHC class I molecules in vivo had been considered improbable, because the defined cellular pathways of MHC class I antigen presentation require that the antigen be expressed endogenously (Bevan, 1976). However, recent experiments designed to determine which cell type presents tumor-specific antigens to MHC class I-restricted CD8 T cells may be quite analogous to the CTL priming that occurs with an in vivo transfected myocyte: In both cases, the antigen is initially expressed exclusively by a nonprofessional APC. In the case of tumor vaccines, Huang et al. (1994) used a model system in which a specific antigen (influenza NP) with known MHC class I epitopes is expressed by a tumor. Parent F1 chimeras, in which the MHC haplotypes of the bone marrow-derived cells either did or did not match the MHC haplotype of the tumor cell were vaccinated with NP-expressing tumors. In all cases, the NP-specific CTL generated were specific exclusively for epitopes presented by MHC alleles expressed by the bone marrow-derived cells, not the tumor cell. Thus, the priming of MHC class I-restricted responses involved the transfer of that antigen to a host bone marrow-derived cell before its presentation to CD8+ T cells. Using subsets of macrophages and defined conditions in vitro, exogenous antigens have recently been shown to enter the MHC class I processing pathway (Kovacsovics-Bankowski and Rock, 1995).

The most direct evidence implicating bone marrow-derived APCs in the priming events of naked DNA vaccines comes from a recent study by Ertl and colleagues. Using the murine rabies model, they demonstrated that coinjection of naked DNA encoding murine granulocyte-macrophage colony-stimulating factor (GM-CSF) with the gene encoding rabies glycoprotein enhanced both antibody and cellular responses to the rabies glycoprotein antigen (Xiang and Ertl, 1995). The enhanced immunologic responses correlated with enhanced protection against challenge with the rabies virus in vaccinated animals. These results appear analogous to findings that tumor vaccines genetically modified to express GM-CSF provide enhanced systemic immunity against challenge with lethal doses of unmodified tumor cells injected distant to the vaccine site. The proposed mechanism by which paracrine GM-CSF elaboration enhances antigen-specific immune response relates to the ability of this cytokine to induce the differentiation of hematopoetic progenitors into

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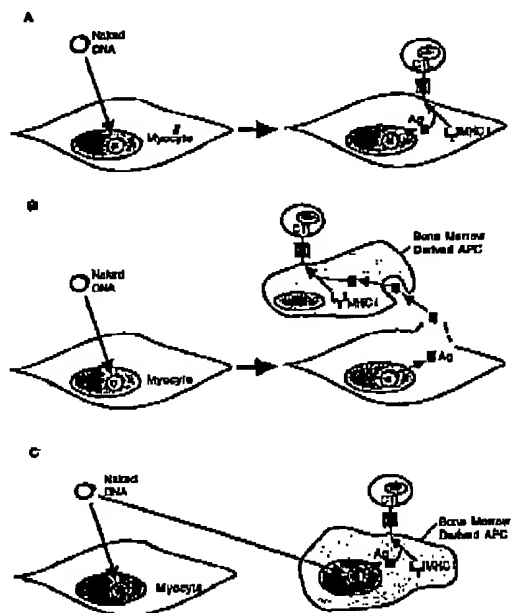
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Figure 2. Three Possible Models for Priming of MHC Class I–Restricted CTL Responses by Intramuscular DNA Injections

(A) Direct presentation by transduced muscle cells.
(B) Transfer of protein antigens to bone marrow–derived APCs with crossover into the MHC class I-processing pathway.
(C) Uptake of DNA directly into bone marrow–derived APCs infiltrating the muscle followed by classical MHC class I processing.

"professional" APCs. In addition to macrophages, GM-CSF has been shown to induce the differentiation and maintenance of dendritic cells. This cell type, which is 2–3 three logs more potent on a per cell basis than macrophages in activating naive T cells in vitro, has been proposed to be the critical APC in initiating immune responses in vivo. Qualitative and quantitative differences in APC composition or traffic into different tissue types may, in fact, account for the lower amount of DNA necessary for intradermal vaccination. In particular, the presence of Langerhans cells in the epidermis provides a ready source of APCs at the injection site.

Another model for immunologic priming by naked DNA vaccines bears consideration since it has neither been ruled in or ruled out. While Sutton's law dictates that attention be focused on the myocyte in the case of intramuscular DNA vaccines, it is certainly possible that small numbers of infiltrating bone marrow–derived APCs are themselves directly transfected (Figure 2C). Because these bone marrow–derived cells are motile, while myocytes are stationary, they may have fled the scene by the time the biopsy is taken, thus giving the appearance that myocytes are the only transfected cell. While small in number, such transfected bone marrow–derived APCs may ultimately be the critical players in priming immune responses in draining lymph nodes.

Now that naked DNA has become established as a clear player in the vaccine field, it will be important to dissect the mechanisms by which it activates immune responses. It is only through these studies that intelligent modifications can be introduced to maximize both qualitatively and quantitatively its ultimate potency.

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PATENT

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DNA vaccination against virus infection and enhancement of antiviral immunity following consecutive immunization with DNA and viral vectors

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Summary Recent demonstrations of the immunogenicity of antigens encoded in DNA plasmids following delivery by various routes have heralded a new era in vaccine development. In this article, we review progress in DNA-based antiviral immunoprophylaxis. Preclinical studies have already established the immunogenicity of DNA plasmids encoding protective antigens from a wide variety of viral pathogens and work published in recent months has raised real prospects of broadly protective DNA vaccination against infections with influenza virus and HIV. We also describe a consecutive immunization protocol consisting of a priming dose of vaccine antigen encoded in DNA plasmids followed by a booster with the same antigen encoded in recombinant fowlpox virus vectors. We have used this strategy to generate protective antiviral cell-mediated immunity and sustained, high-level antibody responses both systemically and at mucosae, and to elucidate immunological mechanisms underlying the development of immunity to antigens delivered in DNA vectors.

Key words: consecutive immunization strategy, DNA vaccines, virus infection.

Introduction

In the early 1990s it was demonstrated that genes encoded in DNA plasmids were expressed in mice following either i.m. injection into muscle tissue[1] or particle-mediated bombardment of skin via a 'gene gun'.[2,3] These observations provided the foundations for an explosion of research activity leading to the development of novel possibilities for vaccination against disease. Studies in animals have shown that DNA vaccines are capable of eliciting persistent humoral and cell-mediated immune responses to a wide range of viral, bacterial and parasitic antigens, many of which are protective against infection with the native pathogen, as well as effective tumour-specific immunity and, in some cases, work has progressed to the stage of phase I clinical trials for human vaccines. The first demonstration of the protective efficacy of DNA vaccination was against infection with influenza virus[4] and many subsequent studies have shown that this approach has great potential for the development of improved immunoprophylaxis against a wide range of viral pathogens.

DNA vaccination against virus infections

Influenza virus

Ulmer and colleagues first showed that mice immunized with DNA plasmids encoding the nucleoprotein (NP) of influenza virus generated NP-specific serum antibody and memory

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CTL responses and were protected against morbidity and mortality when challenged with heterotypic influenza virus.[4] Subsequent studies in mice, ferrets and chickens confirmed that DNA encoding haemagglutinin (HA) or NP genes generated immune responses which protected against disease. Long-lived, protective antibody responses against HA[5,6] and cross-protective CTL against epitopes of NP,[7] including both immunodominant and immunorecessive determinants,[8] were generated in animals given DNA via the i.m. route. Gene gun delivery of similar plasmids also stimulated long-term antibody responses against HA which conferred resistance to infection with homologous strains of influenza virus,[9,10] but which also provided protection against lethal antigenic variants that differed from the primary antigen by as much as 13% of their amino acid sequence.[11,12] These studies suggest that DNA vaccination with appropriate antigens may provide broad protection against antigenic drift as exhibited by influenza virus. Protection against lethal challenge has also been achieved in chickens following direct mucosal (intranasal) or i.v. administration of DNA encoding HA, albeit less efficiently than gene gun delivery, demonstrating the breadth of routes of effective immunization which may be possible using this technology.[9] The first preclinical trial of a prototype DNA vaccine against influenza virus infection was reported only 2 years after the initial demonstration of the protective efficacy of this approach.[13] The vaccine constructs used in these studies encoded a cocktail of surface (HA) and internal (NP and M1) proteins from several different strains of the virus. In ferrets and non-human primates given two i.m. doses of the vaccine both CTL and haemagglutination-inhibiting antibodies were induced and provided levels of protection against an antigenically distinct strain of influenza virus which compared favourably with those induced by licensed inactivated vaccines.

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Human and simian immunodeficiency virus

The first reports of successful DNA vaccination for immune responses against HIV appeared shortly after the work of Ulmer and colleagues in the influenza model. Antibody and CTL responses against HIV and simian immunodeficiency virus (SIV) proteins encoded in DNA vaccines, including gp160, gag and nef, were reported in mice and non-human primates which had been immunized i.m. or via the gene gun.[14-18] It is noteworthy that concomitant vaccination with plasmids expressing IL-12 or granulocyte/macrophage colony-stimulating factor (GM-CSF) had the effect of boosting HIV-specific CTL and antibody responses, respectively,[19] pointing to the broad potential of cytokine co-expression in stimulating DNA vaccine-induced immune responses, as previously shown with other vector-based vaccines.[20,21] An experimental vaccine comprising DNA plasmids encoding five different forms of SIV proteins and delivered i.m. and by gene gun (or by either of these routes alone) elicited transient neutralizing antibody responses and persistent specific CTL, but failed to prevent infection or loss of CD4+ T cells upon challenge with virulent SIV.[22] More recently, it has been reported that two chimpanzees given eight i.m. inocula of plasmids encoding env, rev and gag/pol of HIV-1 developed specific cellular and humoral responses and were protected from infection with heterologous virus grown in chimps, unlike an animal that had been immunized with control DNA.[23] Although the immune responses elicited by this vaccination protocol were not reported in detail and the numbers of animals used were small, it was clear that viraemia in the protected animals was delayed and transient and they remained negative for evidence of infection, as assessed by polymerase chain reaction (PCR), for 1 year after challenge. While the great discrepancies between the results of these two studies remain to be resolved, they could, for example, be related to differing DNA vaccine preparations and immunization protocols. It is clear, nevertheless, that the technology of DNA vaccination increases the chances of effective immunoprophylaxis against HIV.

Other viral diseases

Attempts to develop DNA vaccines against virus infection have by no means been confined to influenza virus and HIV. One of the earliest demonstrations of the ability of DNA vaccination to elicit protective antibody responses was against bovine herpesvirus.[24] Herpes simplex virus (HSV) antigens, gB and gD, encoded in vaccine plasmids have also been shown to induce protective antibody responses in guinea-pigs and mice when given i.m.,[25-27] while mucosal delivery induced antibodies which provided less effective protection against challenge.[28] Papillomavirus is another agent which, like HSV, is a sexually transmitted pathogen causing recurrent disease which may progress to cancer. A recent report describing the development of a DNA vaccine which elicits neutralizing antibodies against the major viral capsid protein and which confers protection against challenge in rabbits is therefore of great potential significance.[29] Recent attention has also focused on the development of DNA vaccination strategies against hepatitis B and C viruses. Hepatitis B surface proteins encoded in DNA elicit high titre

specific antibodies following i.m. delivery in mice[30] and chimpanzees,[31] while co-expression of IL-2 in the vaccine plasmid led to a dramatic enhancement of both specific antibody and cell-mediated immune responses against these antigens.[32] Highly significant results from studies which focused on mechanisms of immune stimulation by hepatitis B DNA vaccines indicated that these constructs may have the capacity to 'break' immunological tolerance and to evoke broadly cross-reactive immune responses.[33] There is currently no effective vaccine against infection with hepatitis C virus (HCV); however, recent reports of the generation of antibody and CTL responses specific for the HCV core protein expressed in DNA plasmids[34,35] offer some hope in this regard, due to the highly conserved nature of this antigen. Responses against HCV core protein may also be enhanced by co-expression of the genes encoding IL-2 or GM-CSF in the vaccine vector.[36]

Other viruses against which DNA vaccination has been used to generate neutralizing antibodies or other protective immune responses include rabies virus,[37,38] lymphocytic choriomeningitis virus,[39,40] equine herpesvirus,[41] Newcastle disease virus,[42] rotavirus,[43] St. Louis encephalitis virus,[44] murine cytomegalovirus[45] and bovine viral diarrhoea virus.[46]

Consecutive immunization with DNA and viral vectors

We have devised a consecutive immunization strategy involving i.m. priming by DNA vaccination and boosting with poxvirus vectors encoding common vaccine antigens in attempts to generate improved specific immune responses. The viruses used in these studies were recombinant vaccinia viruses (rVV) and fowlpox viruses (FPV), which we have previously developed as vectors for the induction of long-lasting immune responses to heterologous vaccine antigens.[20,47] These responses have been enhanced, in many cases, by the co-expression of genes encoding cytokines.[20,21,47] The rationale behind this consecutive vaccination strategy was that DNA immunization, which elicits low-level but persistent immunity, may prime for greatly enhanced responsiveness following boosting with another persistent vector such as FPV, which expresses somewhat greater levels of vaccine antigen. In addition, immune responsiveness is likely to be directed almost entirely against the encoded vaccine antigens as the vectors themselves, which do not replicate, elicit poor responses. In the following studies, mice were primed with 100 μg DNA and boosted with 10⁷ p.f.u. 2 weeks later, unless otherwise stated.

Systemic immune responses

In the first instance, mice given an i.v. booster inoculum of rFPV encoding the HA gene of influenza virus (FPV-HA) 4 weeks after i.m. immunization with DNA vaccine (pCMV/HA) exhibited high levels of anti-HA antibody within 1 week of boosting (Table 1). Specific antibodies were predominantly of the IgG2a subclass. Antibody titres peaked at high levels (over 1 mg/mL) by 3 weeks post boosting and were maintained at significant titres for at least 15 weeks. The level of responsiveness was dependent on the priming dose of

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Table 1   Systemic anti-HA antibody responses after consecutive immunization with pCMV/HA and FPV-HA

	Anti-HA antibody (μg/mL) after boosting with FPV-HA			
	pCMV/control	pCMV/HA		
Time post boosting (week)	100 μg*	100 μg†	10 μg†	1 μg*
1	16.0 ± 3.5	533.6 ± 151.9	433.5 ± 400.4	19.1 ± 6.6
3	18.3 ± 1.4	1061.4 ± 347.5	510.8 ± 42.5	22.9 ± 2.0
6	8.3 ± 2.9	559.5 ± 217.8	373.9 ± 31.2	17.5 ± 5.4
15	13.2 ± 1.4	235.2 ± 122.2	197.0 ± 16.9	9.3 ± 4.6

*Titres in control groups that were boosted with control virus not expressing HA (FPV-M3) were < 0.05 μg/mL.
†Titres in control groups that were boosted with control virus not expressing HA (FPV-M3) were 2.2–18.2 μg/mL.

pCMV/HA. While this strategy was highly effective for the induction of specific antibody responses, primary splenic anti-HA CTL responses were not found in mice primed with pCMV/HA and boosted with FPV-HA (data not shown), although moderate CTL responses were detected when splenocytes were taken from mice primed with pCMV/HA 4 weeks earlier and restimulated for 5 days with influenza virus *in vitro* (Table 2).

Mucosal immune responses

Vaccination for mucosal immunity has been notoriously difficult to achieve, particularly via systemic immunization. Previous reports, however, have indicated that systemic DNA vaccination may prime for immune responses at mucosae.[4,9] We have primed mice with pCMV/HA via the i.m. route 4 weeks prior to intranasal boosting with FPV-HA in attempts to elicit strong, sustained mucosal responses. Although specific antibody-secreting cells (ASC) were not detected in the lungs of mice given pCMV/HA only (data not

shown), both mucosal anti-HA IgG (particularly IgG2a) and IgA antibody responses were markedly enhanced in DNA-primed animals given FPV-HA and were sustained for at least 3 weeks (Table 3). These augmented responses were further elevated in mice boosted with FPV-HA which co-expressed IL-6 (Table 3). Priming with pCMV/HA via gene gun immunization prior to intranasal delivery of FPV-HA was similarly effective for the enhancement of mucosal immune responses (data not shown).

We have previously shown that primary anti-HA CTL responses were generated in the lungs following intranasal immunization with FPV-HA,[47] however, we could find no evidence for enhancement of local CTL activity in mice given pCMV/HA and FPV-HA consecutively (data not shown).

Protection against challenge with influenza virus

We have also studied the degree of protection against morbidity and mortality afforded by our consecutive vaccination

Table 2   Secondary systemic antigen-specific CTL responses after DNA vaccination

	% Specific lysis of L929 target cells infected with			
% Restimulated culture	Uninfected	Influenza virus	FPV-M3	FPV-HA
100	25.85	59.17	27.00	48.87
33	9.47	49.94	22.11	29.73
10	3.39	28.80	15.47	10.74
3	0.85	13.14	7.98	6.53

Data shown are mean values from triplicate wells. SEM were < 5% and are omitted for clarity. The data shown are from a single experiment representative of three such experiments.

Table 3   Mucosal anti-HA antibody responses after consecutive immunization with DNA and recombinant FPV

			No. anti-HA ASC/10⁶ cells (mean ± SD)*		
Time post boosting	Immunization	Boosting	IgG1	IgG2a	IgA
Week 1	pCMV/HA	FPV-HA	< 2	382.2 ± 46.8	195.6 ± 53.9
	pCMV/HA	FPV-HA-IL6	93.9 ± 13.3	1324.4 ± 93.7	768.9 ± 46.8
	pCMV/control	FPV-HA	< 2	145.5 ± 10.5	60.6 ± 21.0
	pCMV/control	FPV-HA-IL6	< 2	359.8 ± 111.5	189.0 ± 40.2
Week 3	pCMV/HA	FPV-HA	< 2	135.0 ± 15.0	282.5 ± 23.3
	pCMV/HA	FPV-HA-IL6	25.0 ± 5.0	540.0 ± 63.8	793.8 ± 63.4
	pCMV/control	FPV-HA	< 2	< 2	< 2
	pCMV/control	FPV-HA-IL6	6.3 ± 3.6	63.3 ± 20.8	87.5 ± 18.5

*Numbers of anti-HA ASC in control groups that were boosted with control virus not expressing HA (FPV-M3) were < 2/10⁶ cells.

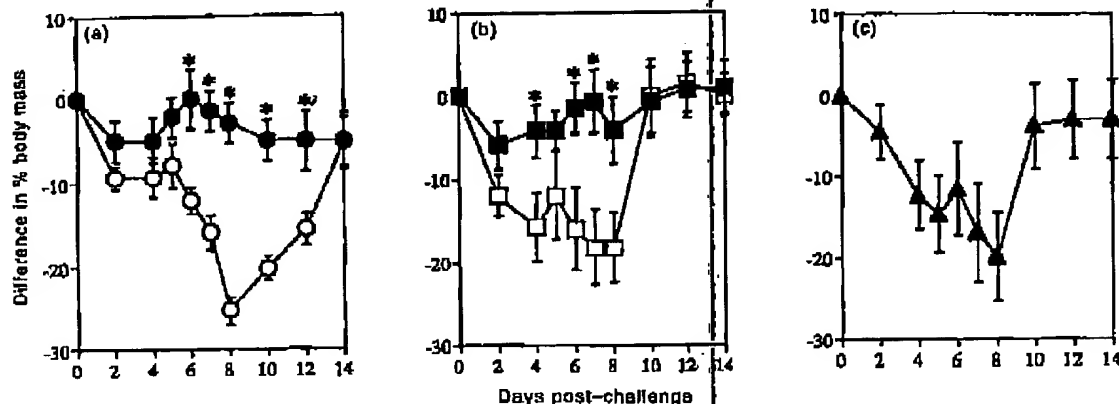No Transmission Information Available   in on line [0] for SF10017 printed 11/20/2002 08:21 * Pg 52/62
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Figure 1   Morbidity in mice consecutively immunized with DNA and fowlpox (FPV) virus after challenge with influenza virus. Groups of six CBA/H mice were immunized with (a) (O) 100 μg of pCMV/control and 10⁷ plaque-forming units (p.f.u.) of FPV-HA i.v. or (●) 100 μg of pCMV/HA and 10⁷ p.f.u. of FPV-HA i.v. (b) (□) 100 μg of pCMV/control and 10⁷ p.f.u. of FPV-HA intranasally or (■) 100 μg of pCMV/HA and 10⁷ p.f.u. of FPV-HA intranasally. Mice were boosted with FPV-HA 4 weeks later and challenged with 100 LD₅₀ of mouse-adapted influenza virus 1 week after boosting. Mice immunized with (c) (▲) 100 μg of pCMV/HA only were challenged at 4 weeks post immunization. Morbidity was recorded as differences in percentage of body mass at different time points after challenge compared to the original body mass recorded on day 0. Data shown are the mean ± SEM of percentage of mass lost for six mice after challenge. The results are representative of two experiments. *P < 0.05, determined by Student's t-test in comparison with mice given pCMV/control before boosting.

protocols (Fig. 1). Mice given pCMV/HA alone or in combination with FPV-HA were subsequently challenged with homologous influenza virus (100 LD₅₀ given intranasally). All mice given either pCMV/HA or FPV-HA alone or in combination were protected from the lethal challenge, although the former groups displayed transient but significant loss of bodyweight (Fig. 1c). However, no such morbidity, nor any other symptoms of infection, were seen in mice primed via the i.m. route with pCMV/HA and boosted with FPV-HA either i.v. (Fig. 1a) or intranasally (Fig. 1b) prior to challenge with influenza virus.

Mechanisms of protective cellular immunity induced by DNA vaccination

It appeared that the consecutive immunization protocol failed to elicit significant primary specific CTL responses as outlined above (albeit after only a single inoculum of each construct). We therefore used a different strategy to measure the development of protective cellular immunity. It was previously shown that priming of mice with influenza virus generated specific antiviral T cells which were able to control subsequent infection with rVV expressing homologous HA glycoprotein, but not with control VV.[48] We found that rVV encoding HA was rapidly controlled in mice which had been immunized consecutively with pCMV/HA and FPV-HA and that the immune responses mediating clearance of VV persisted for at least 28 days after boosting with FPV-HA (Table 4). T cells and their secreted antiviral factors are known to be important for the clearance of VV.[49-51] In order to study which cells were responsible for the control of VV infection in the present study, we transferred different cell populations taken from mice which had been consecutively immunized into irradiated recipients and challenged the latter

Table 4 Protection against VV challenge following consecutive immunization with pCMV/HA and FPV

Primary immunization	Secondary immunization	Challenge (day)	Virus titres in ovaries (log₁₀ ± SEM)
pCMV/HA	FPV-HA	VV-HA (7)	< 2.0
–	–	VV-HA (7)	7.1 ± 0.2
pCMV/HA	FPV-HA	VV-HA (17)	< 3.0
–	–	VV-HA (17)	6.3 ± 0.7
pCMV/HA	FPV-HA	VV-HA (28)	< 2.7
–	–	VV-HA (28)	6.6 ± 0.6

Table 5 CD4⁺ and CD8⁺ T cells mediate protection against VV challenge following consecutive immunization with pCMV/HA and FPV

Cells transferred	Virus titres in ovaries (log₁₀ ± SEM)
Nil	6.4 ± 0.4
Undepleted	3.4 ± 0.2
CD4⁺ depleted	< 2.0
CD8⁺ depleted	< 2.0
CD4⁺ and CD8⁺ depleted	5.1 ± 0.4

with VV-HA 1 day later. As shown in Table 5, either CD4⁺ or CD8⁺ immune T cells generated following consecutive immunization were sufficient to control the VV infection.

Cell transfer experiments were also used to study the nature of priming for antiviral antibody responses by pCMV/HA. The transfer of immune T cells, but not B cells, from mice primed with pCMV/HA 2 weeks earlier was sufficient to allow the development of high titre anti-HA antibody responses in irradiated recipients by 14 days following chal-

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Table 6 Priming of T cells but not B cells in mice immunized with pCMV/HA

Cells transferred		Antibody titres (± SEM)
T cells	B cells	
Primed	Primed	21 942 ± 6245
Normal	Normal	960 ± 606
Primed	Normal	25 600 ± 15 676
Normal	Primed	3520 ± 2805

Table 7 Antibody titres in interferon-α/β receptor and interferon-γ receptor-deficient mice consecutively immunized with pCMV/HA and FPV-HA

Mice	Anti-HA antibody titres (± SEM)	
	Primary immunization pCMV/HA	Secondary immunization FPV-HA
Wild-type	560 ± 219	78 080 ± 79 463
Interferon-γR−/−	220 ± 178	46 080 ± 11 048
Interferon-α/βR−/−	< 10	9600 ± 3695

lenge with FPV-HA (Table 6). FPV-HA was given 7 days after cell transfer. These data indicate that DNA vaccination, at least by the i.m. route, most effectively stimulates antigen-specific T cells. This may be due to the relatively small amounts of antigen which are expressed in transfected tissues following immunization with these constructs.

The influence of cytokines in the development of immunity following DNA vaccination

We have used mice rendered genetically deficient for a range of cytokines or their receptors in order to assess the role of these factors in the development of antiviral antibody responses following vaccination with pCMV/HA either alone or consecutively with FPV-HA. Mice lacking functional receptors for IFN-γ[52] mounted strong, specific day 18 anti-HA IgG responses to pCMV/HA, and by 7 days following the consecutive vaccination protocol, which were reduced about two-fold in comparison to those seen in wild-type control mice (Table 7). In marked contrast, IFN-α/β receptor knock-out mice[53] were unable to mount significant responses following immunization with the HA plasmid and there was no evidence for enhanced responses following consecutive immunization with FPV-HA. These data suggest that endogenous type 1 interferon activity may be crucial for T cell priming following DNA vaccination. This may be related to the recent observation that the immunogenicity of plasmid DNA may be determined by the presence of immunostimulatory sequences (ISS) containing a particular CpG dinucleotide motif.[54] Plasmids lacking such sequences elicit poor immune responses and the ISS probably act through their capacity to stimulate the transcription of large amounts of type 1 interferons and IL-12.[54] The early expression of these factors may explain the tendency of DNA vaccines to elicit immune responses with a type 1 bias, particularly after i.m. delivery.[55] (AJ Ramsay, unpubl. obs., 1997). Further preliminary studies suggest that neither endogenous IL-4 nor TNF-α are crucial

for the induction of antibody responses following DNA vaccination (data not shown).

Conclusions

DNA vaccination offers great hope for improved immunoprophylaxis against infections caused by a wide range of pathogens, including viruses. Indeed, the first human clinical studies using DNA immunogens have already begun. Our studies in this area have focused on the development of a consecutive vaccination strategy using DNA plasmids and rFPV encoding a common vaccine antigen. Both vectors appear safe and effective but elicit relatively low levels of specific antibody and multiple inocula are usually required to generate satisfactory responses. However, their sequential use elicits antibody levels of the order of those found in convalescent sera, which is remarkable given the small amounts of antigen that are likely to be expressed in the host. A possible explanation lies in the failure of these constructs to elicit significant vector-specific immunity, which minimizes the prospects of antigenic competition and may allow the immune response to be directed almost entirely against the heterologous vaccine antigen. It also appears that DNA vaccines are a particularly effective means of priming for T cell-mediated immunity, possibly due to their expression of relatively small amounts of antigen. Both T cell-mediated antiviral immunity and systemic and mucosal antibody responses were significantly enhanced by the use of this protocol, while the co-expression of IL-6 further increased specific antibody titres. In summary, we have shown that the use of DNA vaccines and rFPV in consecutive immunization regimens represents a highly effective strategy for the induction of sustained antiviral immunity.

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Fowlpox Virus Host Range Restriction: Gene Expression, DNA Replication, and Morphogenesis in Nonpermissive Mammalian Cells[1]

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Fowlpox virus (FPV), type species of the *Avipoxvirus* genus, causes a slow-spreading pox disease of chickens. Following infection of mammalian cells there is no evidence of productive replication of FPV although cytopathic effects are induced and FPV recombinants have been shown to express foreign genes from vaccinia virus early/late promoters. Here we report results of a study to investigate the expression of FPV genes, the replication of FPV genomic DNA, and any ultrastructural changes in mammalian cells infected by wild-type virus, undertaken as a first step in elucidating the nature of the block (or blocks) to productive replication of FPV in mammalian cells. Early and late gene expression as well as genomic DNA replication was observed in fibroblast-like cell lines of monkey and human origin. Furthermore, viral morphogenesis was observed in monkey cells, with the production mainly of immature particles though smaller numbers of apparently mature virus particles were observed.   © 1993 Academic Press, Inc.

Within the poxviruses, host range varies in nature and extent. Within the orthopoxviruses, for instance, cowpox virus (CPV) infects a large number of mammalian species whereas ectromelia virus only naturally infects the mouse (1). Three host range genes (hr) have thus far been identified in the orthopoxviruses: C7L and K1L in vaccinia virus (2, 3), and the CHO hr gene from CPV (4). While vaccinia virus does not need these genes to replicate in Vero cells, the presence of either C7L or K1L is necessary to allow it to replicate in human or pig kidney cell lines. Vaccinia virus is, however, unable to replicate in chinese hamster ovary (CHO) cells unless supplied with the CHO hr gene from cowpox virus. The CHO hr gene can also replace the C7L or K1L genes in vaccinia virus allowing replication in human or pig kidney cell lines. The K1L and CHO hr genes allow replication of vaccinia virus in rabbit kidney cells but the C7L gene does not permit this (3). It has been reported recently that a derivative (NYVAC) of vaccinia virus strain Copenhagen, which has the C7L and K1L genes (as well as 15 other nonessential open reading frames, ORFs) deleted, is able to replicate at a very low level in some human cell lines (5). There are, however, likely to be other genes involved in host range determination in vaccinia virus. The MVA strain of vaccinia virus (6) is apparently restricted to growth in chick embryo fibroblasts (CEFs). It has an intact C7L gene (3)

and a truncated K1L gene (7) but results of experiments to restore mammalian cell tropism suggest that other mutations might be present (3).

In contrast to the wide host range of many orthopoxviruses and despite their larger genome (8), the avipoxviruses are restricted to growth in avian species. While high multiplicity of infection of mammalian cells with fowlpox virus (FPV), the type species of the avipoxviruses, causes cytopathic effects (CPE), there is no evidence of productive replication of the virus in those cells. There has been only one report of the isolation of an avipoxvirus from a mammal: in 1969, a virus was isolated from an already terminally ill, and probably immunosuppressed, rhinoceros held in a zoo (9). The isolate was identified as a somewhat atypical FPV, based on pathological, virological, and serological data (10).

While there is little known about the fate of avipoxviruses in mammalian cells, it has been shown recently that recombinant FPV and recombinant canarypox viruses, expressing the rabies virus glycoprotein or the measles virus F and HA glycoproteins under the control of the early/late vaccinia virus H6 promoter, can express those genes in mammalian cells (11–13). Furthermore, antibodies could be raised against the recombinant proteins when several mammalian species were inoculated with the recombinant viruses and, moreover, protection was observed upon appropriate challenge with rabies or with canine distemper viruses (11–13). While these somewhat surprising results using foreign genes under the control of vaccinia promoters do not shed light on the nature or extent of avipoxvirus gene expression and replication in mam-

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malian cells, they do indicate that the block to avipox-virus replication in mammalian cells is not at the level of attachment of the virus and penetration into cells.

The aim of this study was to determine what steps of the FPV replication pathway were active (and at what levels) in mammalian cells of various types. This would allow us to discriminate between two possible explanations for the lack of FPV replication in mammalian cells, either: (i) that the block to replication might be attributed to failure of a particular step (such as DNA replication or late gene expression, hereafter referred to as "postreplicative gene expression") or (ii) that the low efficiency of a number of successive steps might lead to breakdown of the overall process. Thus it was necessary to determine whether expression of viral genes was limited only to early gene expression or whether postreplicative gene expression also took place. While the occurrence of postreplicative gene expression would indicate that DNA replication had taken place, it was necessary to determine if DNA replication occurred in case there was a block between DNA replication and postreplicative gene expression. If DNA replication and postreplicative gene expression occurred, it would be necessary to see if ultrastructural changes occurred within the cell and whether such changes were normal or abnormal.

We confirmed previous observations of others (13) that following infection of Vero cells with FPV grown on CEFs, at a multiplicity of infection (m.o.i.) of about 50 plaque forming units (PFU) per cell, there is no evidence of production of infectious virus. Neither intra-nor extracellular virus could be detected (by plaque assay on CEFs) after three passages, each of 7 days, on Vero cells. In addition there was no sign of CPE after the first passage.

Viral protein expression was examined by SDS–polyacrylamide gel electrophoresis of extracts of infected cells pulse-labeled with [³⁵S]methionine. Initial experiments using FPV-infected CV-1 cells showed considerable expression of what appeared (by their time of appearance and abundance) to be postreplicative viral polypeptides. Another monkey cell line, Vero, showed almost identical results and the polypeptides were confirmed as postreplicative as their expression was shown to be sensitive to cytosine arabinoside (Ara C, Fig. 1). In Vero cells, expression of polypeptides of molecular weight 27K and 36K was clear (Fig. 1, compare lanes 7 and 8 with lanes 9 and 10), being detectable from 24 hr postinfection (p.i.). Expression of a polypeptide of molecular weight 85K was also postreplicative. A 39K polypeptide was expressed both early (4 hr) in an Ara C-resistant manner and late in an Ara C-sensitive manner. Polypeptides of a similar molecular weight to those expressed late and in an Ara C-sensitive manner in Vero cells (i.e., 27K, 36K, 39K, and 85K) were also seen to be expressed in the same manner in

permissive CEFs. Expression of other postreplicative polypeptides was also observed in CEFs, such as those of molecular weights 42K, 60K, and 85K (Fig. 1). The only early viral polypeptides seen to be expressed in infected CEFs in an Ara C-resistant manner were the 39K early/late polypeptide seen in Vero cells and another early/late polypeptide (42K).

In a human cell line, MRC-5, a number of postreplicative polypeptides were expressed, in an Ara C-sensitive manner, though only from 36 hr p.i., including polypeptides of molecular weights 27K, 39K, 42K, and 64K (Fig. 1). Of these, the 39K and 42K polypeptides were also expressed early (from 4 hr p.i.) in an Ara C-resistant manner. Several early, Ara C-resistant polypeptides were also observed, notably those of 21K and 28K. Following FPV infection of another human cell line, HeLa, there was no sign of expression of postreplicative viral proteins, although expression, in an Ara C-resistant manner, of early polypeptides (39K, 42K, and 52K) was observed (data not shown).

We also assayed the abundance of the 39K immuno-dominant surface protein encoded by the 34K ORF, by Western blotting lysates of infected cells using anti-serum raised against a peptide derived from a repeat region within the protein (14). The protein was expressed as an early gene but it appeared to be more strongly expressed as a postreplicative gene in both CEFs and Vero cells, being observed late in infection only in the absence of Ara C. In MRC-5 cells some protein was still observed in the presence of Ara C late in infection but this was less than in the absence of Ara C (data not shown), showing that postreplicative gene expression was probably occurring.

It was possible that postreplicative gene expression in mammalian cells is a peculiarity of the FP9 strain of FPV, due to its extended passage history in CEFs. HP438, the source of the FP9 plaque-purified strain, had been passaged 438 times (15). We therefore used a recombinant of the chick-embryo-derived Duphar "poxine" strain of FPV, which encoded β-galactosi-dase, to assay late gene expression in CEFs and Vero cells in the presence and absence of Ara C. In this recombinant virus, the lacZ gene is inserted down-stream of the FPV 4b promoter (16) within ORF1 of the terminal 6-kb BamHI fragment (17). The β-galactosi-dase activity in extracts of infected cells was determined as previously described (16). The results (data not shown) demonstrated clear expression of β-galac-tosidase activity in CEFs and Vero cells infected by the poxine recombinant but expression was only detected in the absence of Ara C. The level of β-galactosidase activity in Vero cells relative to CEFs was 27% at 24 hr p.i. and 67% at 48 hr p.i. Thus we conclude that post-replicative gene expression by FPV in mammalian cells is not restricted to the FP9 strain.

Expression of postreplicative genes in infected
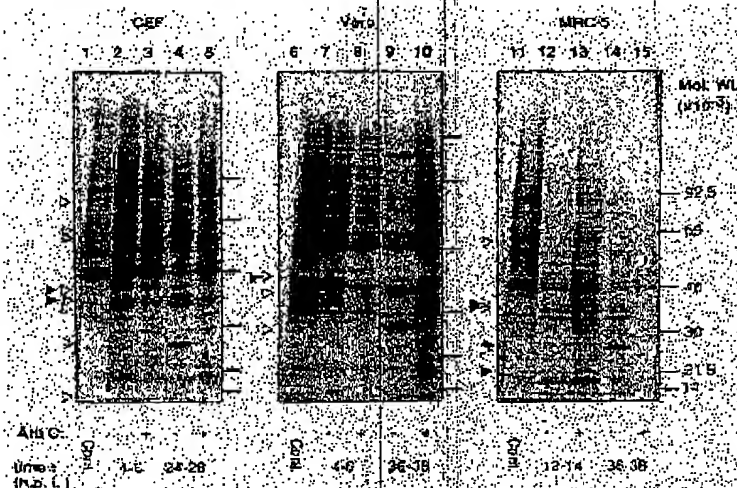
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Fig. 1. Analysis of protein synthesis in cells pulse-labeled with [³⁵S]methionine. Flasks of cells were infected (or mock-infected, "Cont.") with the same amount of FPV strain FP9 for 1 hr, at an m.o.i. of about 10 PFU per cell for CEFs and 40 to 50 for Vero cells. The cells were washed, the medium was replaced, and then the cells were incubated for the indicated times (hr postinfection, h.p.i.), at which point they were harvested and resuspended in methionine-free medium containing 2% dialyzed fetal calf serum and 20 μCi/ml [³⁵S]methionine. Following incubation at 37° for 2 hr, the cells were harvested, lysed in sample buffer, and subjected to electrophoresis on 7.5 to 16% SDS-polyacrylamide gradient gels. For each time point, duplicate samples were prepared, one from cells incubated in the presence (+) of Ara C (40 μg/ml from 1 hr before infection) and one from those incubated in normal medium (−). The position of molecular weight markers is indicated. The major viral early and postreplicative polypeptides, discussed in the text, are indicated by closed and open arrowheads, respectively (polypeptides expressed early and late have both markers). Panels show results for CEFs (lanes 1–5), Vero cells (lanes 6–10) and MRC-5 cells (lanes 11–15).

mammalian cells indicated that viral genomic DNA replication was occurring in those cells. Amplification of DNA was tested directly by dot blotting total DNA isolated from infected cells on to nylon membranes and probing with a FPV-specific DNA probe. Virus DNA replication, inhibited by Ara C, was clearly demonstrated in infected CEFs (Fig. 2a), Vero (Fig. 2b), CV-1 (data not shown), and MRC-5 cells (Fig. 2c).

We have also followed the RNA transcription of early and postreplicative genes using Northern blotting. Probing Northern blots of infected CEFs, Vero, and MRC-5 cells with radiolabeled pUSEP2.1 showed the presence of a 1.7 kb early RNA, expressed in the presence of Ara C, at 4 hr (or 12 hr) and, at lower levels, at 24 or 36 hr (Fig. 3). The probe used also hybridized to typical postreplicative transcripts which were larger, heterogenous in size (2–10 kb) and were expressed in an Ara C-sensitive manner from 12 hr p.i. in CEFs and Vero cells but not at all in MRC-5 cells (Fig. 3). Postreplicative RNA transcripts were detected in MRC-5 cells, however, when a probe covering the genes encoding FPV 4b and the 39K immunodominant protein was used (data not shown).

These results show that FPV early gene expression and DNA replication in monkey (Vero and CV-1) cells occurs at similar times postinfection and at similar levels as compared to CEFs. Thus there appears to be no

block to those processes in monkey cells. In these same cells, postreplicative gene expression takes place but it is retarded and at reduced levels. In human MRC-5 cells, FPV early gene expression and DNA replication also take place but at lower levels than in CEFs, Vero, and CV-1 cells. Expression of some postreplicative genes takes place in MRC-5 cells, but it is even more retarded and at lower levels than is seen in mon-
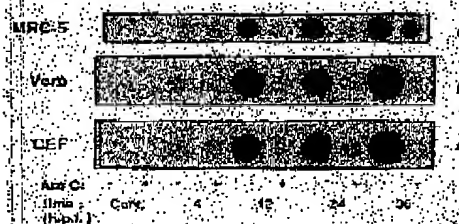

Fig. 2. Dot blot analysis of FPV genomic DNA replication in infected cells. Cells infected (as described in the legend to Fig. 1) or mock-infected ("Cont.") cells were incubated for the times indicated in the presence (+) or absence (−) of Ara C and then they were harvested. DNA was extracted from them (by sonication followed by RNase digestion, proteinase K digestion, phenol-chloroform treatment and ethanol precipitation), applied to a nylon membrane, and hybridized with a randomly-primed [³²P]-labeled FPV DNA clone (pMB369 [25], containing a 3.6-kb DraI fragment of FPV DNA). Panels show results for (a) CEFs, (b) Vero, and (c) MRC-5 cells.

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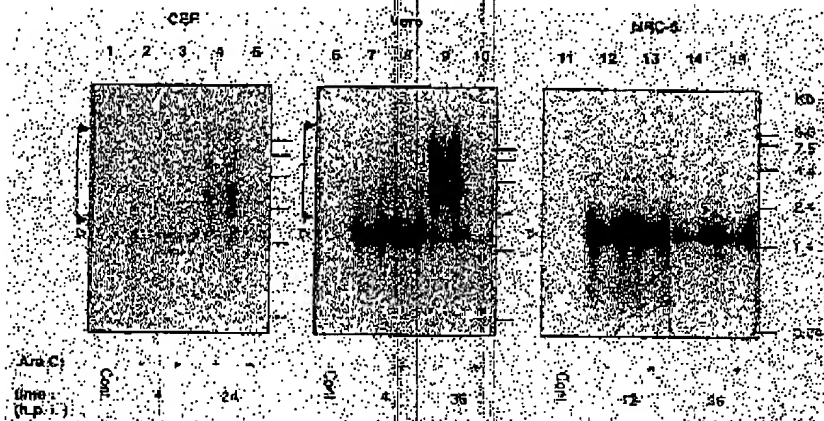

FIG. 3. Northern blot analysis of gene expression in FPV-infected cells. Cells infected (as described in the legend to Fig. 1), or mock-infected ("Cont."), were incubated for the times indicated in the presence (+) or absence (−) of Ara C, then they were harvested and total RNA was isolated (26). The RNA was subjected to electrophoresis on a 1.2% formaldehyde agarose gel and, following Northern transfer, was hybridized to a [³²P]-labeled FPV DNA probe (pUSE2.1, containing a 3.7-kb EcoRI FPV DNA insert; J. Campbell, personal communication) which spans FPV early and postreplicative genes. The location of molecular weight markers is indicated. The position of a 1.7-kb early gene transcript is indicated by an open arrowhead and that of heterogenous postreplicative gene transcripts by tied, closed arrowheads. A sample from uninfected control cells is indicated by Cont. Panels show results for CEFs (lanes 1–5), Vero cells (lanes 6–10), and MRC-5 cells (lanes 11–15).

key cells. Studies with HeLa cells consistently showed a very low level of genome replication and postreplicative gene expression, barely above basal levels (data not shown), a result similar to that seen for postreplicative expression of β-galactosidase in FPV-infected human 143B cells (18). Thus HeLa cells could be considered to show a more extreme example of the delay (and reduction) in postreplicative gene expression seen in MRC-5 cells compared to the monkey cells and in the monkey cells compared to avian cells. It is possible that the lack of an FPV equivalent of either of the vaccinia host range genes, C7L and K1L, accounts for the differences in expression seen between the monkey and human cells, since the presence of one of these genes is necessary for vaccinia virus replication in MRC-5 but not in Vero cells (3).

The role of the morphological cell-type as opposed to the species of origin of the cell lines has not yet been investigated but it should be noted that Vero, CV-1, and MRC-5 cells, which supported significant postreplicative gene expression, are fibroblast-like cell lines whereas HeLa cells, which showed barely any such gene expression, are epithelial-like cells.

As poxvirus DNA replication is known to occur in particular structures within infected cells (known as B-type inclusion bodies, virus factories, or virosomes) we examined infected mammalian cells for changes in cellular ultrastructure. By immunofluorescent microscopy, using chicken anti-FPV hyperimmune serum, at least 85% of infected, but not uninfected, CEFs and Vero cells showed strong cytoplasmic immunofluorescence as early as 12 hr p.i. (data not shown). This was

supported by analysis using a FACScan (Becton Dickinson), in which infected or uninfected, permeabilized or nonpermeabilized cells were labeled with chicken anti-FPV hyperimmune serum (or normal control serum) and subsequently with FITC-labeled anti-chicken Ig second antibody. These analyses showed that essentially all of the Vero cells were infected (data not shown), determined at 24 hr p.i., whether virus was allowed to adsorb for 1 or 12 hr at 37°. Results for fluorescent microscopy showed that the fluorescence was generally localized in a few sites per cell in CEFs at 12 hr p.i., and in Vero cells at 12 and 24 hr p.i. By 24 hr p.i. in CEFs and by 36 hr p.i. in Vero cells, fluorescence was much more extensive and more widely distributed, associated with granular, "phase-dark" regions of the cell.

Electron microscopy was performed to investigate the detailed ultrastructure of FPV-infected monkey cells. The presence of viral nucleoplasm surrounded by "crescent membranes" and immature viral particles was clear in infected Vero (Fig. 4) and CV-1 cells (data not shown) from 24 hr. The "crescent membranes" were typical of those of poxviruses: dense, thickened membranes enclosing viral nucleoplasm. Smaller segments of membrane were also observed around the nucleoplasm: clearly double-layered and resembling squashed tubes in cross-section (Fig. 4), with the side nearer to the nucleoplasm usually thickened. Their double-membrane structure can be explained by the recent report that vaccinia virus particles are formed by enwrapping of viral DNA by a membrane cisterna, derived from a cellular membrane compart-
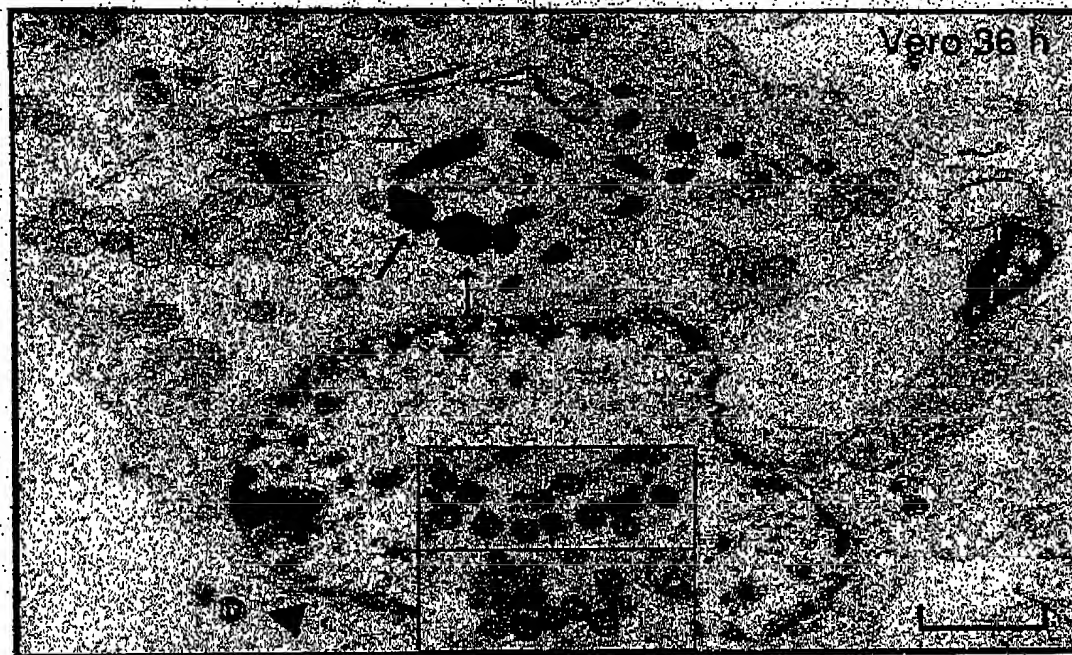
FIG. 4. Electron microscopy of FPV-infected Vero cells. Cells were harvested 36 hr after infection with FPV FP9 (adsorbed for 1 hr, at an m.o.i. of 40 to 50 PFU per cell), fixed using an equal volume of 0.2 M collidine buffer containing 4.5% glutaraldehyde, pelleted, postfixed in 1% osmium tetroxide, dehydrated with ethanol, and embedded in Araldite. Thin sections were stained sequentially with uranyl acetate and lead citrate and were then inspected by transmission electron microscopy. The size marker indicates 1 μm. The micrograph shows pools of nucleoplasm (N) surrounded by viral crescent membranes and immature spherical particles. Also shown are small, "squashed tube" membranes around nucleoplasm (dashed arrows), "filaments" (open arrowheads), "paracrystaline arrays of DNA" (arrows) and a mature membrane-bound particle (closed arrowhead). Insets show immature particles with early-condensing nucleoids, at the same magnification.

ment between the endoplasmic reticulum and the Golgi, thus acquiring two membranes at once (19).

In addition to the virus particles, two types of unusual structures were observed at the sites of virus morphogenesis: long filaments were frequently seen while large dense blocks were rarer (Fig. 4). At higher magnification, it was apparent that both structures were of a repeating, crystalline nature. The filaments were similar to structures described as "filaments with regular periodicity" in FPV-infected cells (20). The blocks resembled the "DNA paracrystals" seen in cells infected by a temperature-sensitive mutant of vaccinia virus, defective in morphogenesis at the non-permissive temperature (21), due to a lesion in ORF D13L (22).

Early-condensing nucleoids were frequently observed within immature particles in the monkey cells (Fig. 4, insets) and mature intracellular naked virus were occasionally seen (data not shown). Mature extracellular enveloped virus with classical poxvirus morphology were also observed at a low frequency, though these were usually membrane-associated (Fig.

4). We have yet to determine whether the latter represent virus synthesized in the mammalian cells or residual virus from the inoculum. All of the virus structures described above were as seen in infected CEFs.

Thus we have demonstrated that FPV is capable of efficient infection of mammalian cells and expression of not only early genes but also late genes, in monkey cell lines, and human MRC-5 cells, at least. In those same cell lines, there is also considerable FPV genomic DNA replication. Viral morphogenesis also readily takes place as far as the formation of immature particles, as was seen for the host range mutant of vaccinia virus, MVA, in human cells (23). Morphogenesis of FPV in monkey cells, however, progresses further in that many of the immature particles show the presence of dense internal nucleoids. Apparently mature particles are also seen, though only rarely.

The high levels of DNA replication that we have observed (particularly in Vero cells) were unexpected and clearly show that there is no major block to FPV DNA replication in these nonpermissive cells. Nor is late gene expression completely blocked (though it is de-

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layed and at lower levels). It is not clear, however, whether the observation that late gene expression is reduced and delayed can by itself account for the lack of production of detectable amounts of infectious virus. It is clear that, in monkey cells, there is sufficient expression to allow FPV morphogenesis to progress at least as far as immature intracellular particles with condensing nucleoids, a step only reached in vaccinia virus morphogenesis more than 5 hr after the release of a block to replication induced by the presence of hydroxyurea (24). The presence of a small number of apparently mature intracellular particles (which may or may not be infectious) also indicates that further maturation can occur. albeit very inefficiently. Thus the overall "block" to FPV replication appears to be the result of at least two relatively inefficient steps: (i) late gene expression and (ii) maturation from the immature particles with condensed nucleoids. It remains to be determined which is rate limiting to the overall process. Clearly there is also a need to purify and characterize, in structural and functional terms, the nature of the FPV particles produced in mammalian cells; this work is in progress.

It will be interesting to see whether the extent of gene expression, replication and morphogenesis of different avipoxviruses in mammalian cells affects their efficacy as recombinant vectors for the expression of foreign antigens in mammals, as significant differences in efficacy have been reported between vectors based on FPV and canarypox virus (11). Our preliminary experiments indicate that upon canarypox virus infection of Vero cells, there is no detectable expression of post-replicative viral genes (P. Somogyi, unpublished).

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